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THE OLFACTORY REACTIONS OF THE COMMON KILLIFISH, FUNDULUS HETEROCLITUS (LINN.)

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In a paper on the olfactory reactions of fishes published in the
eighth volume of the Journal of Experimental Zoölogy (1910), I
have attempted to show that the olfactory organs of catfishes
are stimulated by minute amounts of substance emanating from
materials that can serve these fishes as food; in other words, that
these organs are distance-receptors by which the fishes can scent
out their food much as land animals do. A season at the Biologi-
cal Laboratory of the United States Bureau of Fisheries at Woods
Hole, Massachusetts, enabled me to repeat these tests on the com-
mon killifish, Fundulus heteroclitus, the results of which are given
in this paper. My thanks are due to Dr. F. B. Sumner, Director
of the laboratory, for facilities kindly provided, and to Commis-
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lished.

The olfactory apparatus of the killifish consists of a pair of sacs
each provided with two apertures, one anterior, and the other
posterior. The anterior olfactory aperture is just above the upper
lip and dorsal to the angle of the mouth. It is a small roundish
opening not unlike one of the pores of the lateral-line system
and is on the summit of a low elevation. The posterior olfactory
aperture is an elongated slit somewhat dorsal to the anterior limit
of the eyeball. The mouth of the posterior aperture is partly
occupied by a valve-like fold of skin.

If the quiescent head from a freshly killed Fundulus is examined
in water, no motion is observable about the olfactory apertures.

Suspended carmine is not carried into them or discharged from them; in other words, there is no evidence of a ciliary current passing through the olfactory sacs such as is so easily demonstrated in the catfish. If a head in which the respiratory movements of the gills are still in progress is examined, well marked currents can be demonstrated in the olfactory organs. Suspended carmine is taken in at the anterior aperture and discharged from the posterior one. With each respiratory movement, the valve in the posterior aperture opens, a small amount of water is discharged, and it then closes. This passage of water through the olfactory apparatus is apparently due to the changes of pressure produced by the rythmic activity of the muscles of the gills probably acting in conjunction with valves within the olfactory sacs. The movement of the valve at the posterior aperture follows exactly that of the respiratory apparatus and its automatic character is obvious from the fact that if an anterior aperture in an active fish is closed by having its walls stitched together so that no current of water can enter the sac at that point, the posterior valve of the same side ceases to pulse, though that of the other side continues in normal activity. If, now, the closed aperture is reopened by removing the stitches, the valve previously quiescent begins again to pulse. Thus, though Fundulus has no continuous current through its olfactory sacs, such as the catfish has, it does have a well-developed intermittent current that is not inappropriately designated as respiratory, though this current is in no direct way concerned with the respiratory function. Apparently, as long as the gill muscles of Fundulus carry out respiratory movements, currents of water run through the olfactory sacs.

As a preliminary test to ascertain whether Fundulus could discover hidden food or not, packets of cotton cloth containing dogfish meat wrapped so as not to be visible, and packets made of nothing but cotton cloth were hung in an aquarium in which there were a number of hungry Fundulus. After the packets had been thoroughly soaked in the seawater, the reactions of the fishes to them were watched. The packets without meat were occasionally approached and siezed, but soon dropped. Those that contained meat were sooner or later surrounded by most of

the killifish, which carried on a vigorous competition as to which should have possession of the packet. Frequently the first comer would not only seize the packet and tussle with it, but would often attempt to drive off other fish that had approached the region, attracted apparently by the movements of the first fish. These preliminary tests showed quite conclusively that the normal killifish responds very quickly and in a characteristic way to hidden food.

It was also quite evident from these tests that the killifish, in strong contrast with the catfish, uses its eyes as well as its chemical senses, in seeking and retaining its food. If a small piece of dogfish flesh is dropped into an aquarium in which there are hungry killifish, a fish is almost sure to pounce upon the piece and swallow it quickly. This action is so sudden and begins when the fish is at such a distance from the bit of flesh that it is evidently controlled through the eye. That it is not entirely so, however, is seen from the fact that if a small ball of clean filter-paper is thrown into the water, this too is pounced upon and taken into the mouth but soon discharged. Thus the sight of an object must be followed by an appropriate stimulus of smell or taste, if the object is to be swallowed.

It is the eye, in my opinion, that leads killifish to swim to a packet of plain cloth and seize it even though it contains no food. The fact, however, that the fish do not remain about such a packet long shows how clearly they distinguish it from a packet in which meat is hidden and around which they will gather and tussle for long periods of time. The use of the eye in the preliminary steps of the search for food is shown in the amusing habit that these fish have of chasing drops of water down the glass face of an aquarium as though the drops were bits of food. The eye, then, in Fundulus is serviceable in the initial stages of procuring food, but whether the material is to be persistently nibbled and finally swallowed depends, as the preceding test shows, on other senses than sight.

Another feature in the reactions of these fishes that is of importance in connection with their discovery of food and is probably dependent chiefly on sight, is their habit of seeking food

in schools and not individually. A single fish in an aquarium rarely finds hidden food for the reason that it remains most of the time quietly at the bottom, a position of protection that is assumed by a school of Fundulus when disturbed. If, however, there are a number of fish in the aquarium, they soon rise to the higher water, play about, and thus have a much better chance to run across traces of food. For this reason, I have generally not experimented with single fish, but with small groups of at least five or six.

The part played by the olfactory organs in reactions to hidden food can be determined by first eliminating these organs and then testing the fishes. The olfactory apparatus can be rendered inoperative by cutting the olfactory tracts in a position where they are easily accessible as, for instance, between the eyes. In this situation a small incision can be made through the thin bony roof of the skull and the two tracts can be cut by a single movement of a narrow blade. The first operations of this kind that I carried out were done under ether, but subsequent tests on normal fishes showed that etherization of itself, *i.e.* without any operation, left the fishes in such a condition that they could not distinguish for a number of days packets of cloth without inclosed meat from those that included meat, and, therefore, I was driven to carry out these operations without the use of an anesthetic. Twenty-four hours after such an operation, the fish were fully active, took food, and in all obvious ways seemed normal. When two packets of cloth one with dogfish meat hidden in it and the other without this food, were suspended in the aquarium in which the operated fishes were, these animals nibbled temporarily both packets in a way that made it impossible for an uninformed observer to distinguish one packet from the other. When these two packets were transferred to an aquarium of normal fish, the one containing the food was soon surrounded by a vigorously contesting assembly of fishes, whereas the packet without food was only occasionally nibbled. The evidence from these experiments favors the view that the olfactory organs are necessary to Fundulus in sensing hidden food. The severity of the operations, however, makes this evidence not wholly conclusive.

In order to carry out tests against which the objection could not be raised that the results might be due to the shock of cutting nerves rather than to the loss of a sense organ, the following procedure was employed. By taking two stitches of very fine silk-thread one on either side of the anterior olfactory aperture, it was comparatively easy to close this aperture and thus to prevent any passage of water through the olfactory sacs. Killifish, which previous to the operation gave markedly different and characteristics reactions to the two classes of cloth packets already described, reacted to both kinds of packets after their anterior olfactory apertures were closed, as they had previously done to the packets that contained no food. That this reaction was not to be directly attributed to the operation of stitching up the apertures, was demonstrated in two ways. If, after the stitches were taken, the thread was not drawn up and tied so as to close the aperture, but the ends were allowed to remain free, the fish would react as normal fish do to the two classes of cloth packets, thus showing that the mechanical injury due to the stitches themselves did not influence the fish in any essential way. Further, if fishes whose anterior olfactory apertures had been closed by stitching and tying and whose discrimination for the two classes of packets had thereby been lost, had their olfactory apertures reopened by cutting and removing the thread, they very soon regained their capacity to distinguish packets with food from those without food; in other words, they soon returned to the condition of normal fishes. For these reasons, I believe that stitching up the anterior olfactory aperature is in itself not a disturbing operation for the fish and that the loss of the ability to recognize the presence of hidden food under these circumstances, is in reality due to the loss of the olfactory function. I, therefore, conclude that Fundulus heteroclitus, like the catfish, uses its olfactory apparatus as an organ with which to scent its food; i. e., its olfactory apparatus is a chemical distance-receptor of very considerable importance in its daily activities.

CONTRIBUTIONS TO THE PHYSIOLOGY OF REGENERATION

III.   FURTHER EXPERIMENTS ON PODARKE OBSCURA

SERGIUS MORGULIS

CONTENTS

A. RELATION BETWEEN DEGREE OF INJURY AND RATE OF REGENERATION

The amount of discussion aroused within the last two or three years over the question as to whether or not an additional mutilation of an organism exerts an accelerating influence upon its regeneration testifies to the great significance and interest of the question, especially from the point of view of the regenerative energy.   But unfortunately we are unable to give the final verdict in favor of either alternative.   The idea that by increasing the number of injuries to the organism each injured part is caused thereby to regenerate more rapidly, received recently elaborate evidence from Zeleny ('09a), who was also the first to propound the idea ('03).   The researches of other investigators, however, have more or less failed to accord with his conclusions.   Emmel ('06) observed in the case of the young lobster that the rate of regeneration diminishes with increased mutilation; Scott ('07) found that the rate of regeneration of the fins of Fundulus is independent of the degree of injury; in the brittle-

star Ophicoma pumila I ('09a) found only occasionally a slight increase in the regenerative rate when four or all five arms were removed; finally, Stockard ('09a) concluded from his studies of the medusa Cassiopea and two Ophiurans, that no definite influence is produced in either direction by varying the extent of injury, the rate of regeneration being increased in some species, but either remaining unaffected or even being decreased in others.

In his latest paper concerning the relation between degree of injury and rate of regeneration, Zeleny ('09b, p. 555) modified somewhat his original opinion, declaring that "within moderate degrees of injury a part regenerates more rapidly rather than less rapidly when it has regenerating company than when it regenerates by itself."

I have performed several experiments on Podarke obscura[1] with different degrees of injury in the hope of finding a means of reconciling the opposed views, but the results of the experiments have not justified my expectation. In speaking of "degree of injury," it may perhaps be well to state—since the phrase has been somewhat misunderstood—that I am using it in exactly the same sense as Zeleny did, to indicate the *number* of operations performed upon an animal; and in my experiments worms regenerating tails were decapitated and compared with regenerating worms having their heads intact. Complications arising from the discontinuity of the growth processes, which are a source of serious error in experiments upon crustacea, are entirely absent in the case of worms; furthermore, it is a comparatively easy matter to control the size of the worms used in the experiments, as well as the level of the cut, and by keeping in the same dish both kinds of regenerating worms—decapitated ones and those with heads intact—the greatest possible smilarity of external conditions is secured. So far as differences of sex are concerned, they have no importance in determining the regenerative rate, as will be shown later.

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[1] These experiments were performed in the laboratory of the United States Bureau of Fisheries, Woods Hole, Mass., where I occupied a table during the summer of 1909. Contributions I and II are referred to in the Bibliography under Morgulis 1909b and 1909c.

TABLE 1

Condition of Worms	Decapitated				Heads Intact			
SEGMENTS	OLD	REGENERATED			OLD	REGENERATED		
Date	July 2	July 15	July 22	July 29	July 2	July 15	July 22	July 29
1	9	bud	3	3	16	5	7	7
2	10	5	8	4	17	7	7	8
3	12	5	8	4	17	7	8	8
4	13	3	4	6	17	7	8	9
5	13	4	6	8	17	7	11	12
6	13	5	8	8	18	5	7	7
7	13	8	9	9	18	7	10	11
8	14	2	4	9	19	5	5	5
9	14	7	9	10	20	4	5	5
10	15	bud	2					
11	16	5	5					
Averages ...	13	4	6	6.8	17.7	6	7.6	8

TABLE 2

Condition of Worms	Decapitated				Heads Intact			
SEGMENTS	REGENERATED			OLD	REGENERATED			OLD
Date	July 26	July 30	August 3	July 19	July 26	July 30	August 3	July 19
1	4	6	5	17	3	6	9	18
2	2	7	6	18	4	7	8	19
3	4	4	8	13	5	7	8	17
4	2	6	6	16	4	6	8	16
5	4	5	7	13	3	7	8	16
6	2	5	7	17	4	5	8	16
7	4	5	5	16	3	6	7	18
8	4	4	6	15	5	7	7	15
9	3	3	8	16	4	7	5	16
10	3	5	7	16	5	4	4	18
11	4	4	7	13	4	7		
12	4	5	6	12	3	4		
13	3	7	8	15	4	4		
14	1	5	4	14	3	5		
15	1	7	4	17	2			
16	3	6	6	14				
17	4	5	6	15				
18	4	3	5	17				
19	4	6	4	16				
20	4	6	5	11				
21	4	6	7	9				
22	5	6						
Averages ...	3.32	5.3	6	14.8	3.73	5.9	7.2	16.9

On July 2, 1909, several worms were cut in two at about the middle of the body, and in half of them the heads were likewise removed. The regenerating tails were examined in both sets of worms at regular intervals, and the results are tabulated in table 1.

By the end of two weeks, July 15, the number of segments in the regenerated tails of the worms with heads intact, serving as the control, varied from 4 to 7, the average being 6 segments. In the decapitated worms the number of regenerated segments ranged from 2 to 8, while two of the worms had proliferated only buds of tissue; the average for this group was 4 segments. The difference in the number of regenerated segments in decapitated worms and worms with heads intact was also observed in later stages, as may be seen from the figures under the dates July 22 and 29. It is obvious, therefore, that in this experiment the extra injury caused a decrease rather than an increase in the rate of regeneration of the tail.

A similar experiment was performed on July 19, the results of which are given in table 2. By the close of the first week, July 26, the number of regenerated segments in the worms with head intact varied from 2 to 5 (average 3.73), but in the decapitated worms from 1 to 5 (average 3.32). Ten days and fourteen days after the operation, it will be observed, both the maximum and the minimum number of regenerated segments is lower in the decapitated worms than in the control. Though the depressing effect produced by the extra operation upon the regenerating worms is not as prominent as in the previous instance, yet the results are of the same kind in the two experiments.

The experiments were repeated in a somewhat modified form to find if the result could be changed by inflicting the additional injury when the new tail had already started to regenerate. For this purpose, a number of worms cut in two in the middle of the body were separated into three groups: in the first group, A, the worms were decapitated when the tails were cut off; in group B the worms underwent such an operation a week after the removal of the tail; and the worms of group C, with heads intact, served as control for the experiment. The data are recorded in table 3.

TABLE 3

July 22–August 24

SEGMENTS	OLD	REGENERATED				
Date	July 22	July 29	August 2	August 5	August 8	August 11
Series A. Worms decapitated July 22.	12	2	3	4	4	4
	14	3	3	4	4	4
	15	4	4	5	5	5
	15	4	5	6	6	7 (?)
	15	4	6	6	6	7
	16	4	6	6	6	7
	16	4	7	7	7	7
	16	4	7	7	8	8
	21	4	7	8	8	8
	21		7	8	9	9
Series B. Worms decapitated July 29.	15	4	5	5	5	5
	17	4	5	5	5	5
	17	4	5	5	5	5
	18	4	5	5	6	6
	18	4	5	6	6	6
	18	4	5	6	7	8 (?)
	18	5	6	6	7	9
	19	5	6	7	9	9
	20	5	7	8	9	
	20	5	7	8		
	21	5	7			
Series C. Worms with heads intact.	18	4	4	5	5	5
	18	4	5	5	5	5
	18	4	5	5	6	7
	18	4	6	6	7	7
	19	4	6	6	7	8
	19	4	6	7	9	9
	19	5	7	8	10	10
	20	5	7	8		
	21	6	8	9		

The depressing effect upon the regenerating tails produced by an extra operation can be seen already during the first week. Thus on July 29 in groups B and C, both at this time containing worms with heads intact, the number of regenerated segments varied from 4 to 6, but in group A, containing decapitated worms, the number of regenerated segments varied from 2 to 4. Comparing with each other the corresponding data in groups A and C it will be observed that in the former the worms never regenerated as large a number of segments as are sometimes found in the latter. A scrutiny of the numbers pertaining to group B shows that after the tail had commenced to grow, the removal of the head had no immediate depressing effect upon the regenerating tails.

Thus far the experiments were preformed on worms cut through the middle of the body. The question then arose—Would the results be the same for worms regenerating from a more posterior level? With this in mind, an experiment was performed, similar to the one just described, but in this case only about one-sixth of the worm was cut off. The results of this experiment are reported in table 4.

It will be noticed on glancing over the column under July 29, *i.e.*, one week after the operation, that in this case the number of regenerated segments in all three groups, A, B and C, varies from 3 to 4, the number 3 being, perhaps, predominant in the A-group, or decapitated worms. For the next ten days (till August 8) there is scarcely a change in the condition, and the worms in group B—decapitated after a week's regeneration—show no indication of either an increase or decrease in their regenerative power following the additional operation.

Comparing the results of the last two experiments on worms regenerating from different posterior levels, it is apparent that the additional injury in the anterior region affects the regeneration of the tail the less the more posterior the level of the cut, but that in no case is the tail regeneration accelerated.

Bearing in mind the importance, for purposes of comparison, of following the process of regeneration step by step from the earliest stages (see Morgulis, '09b), I traced the influence of

TABLE 4

July 22–August 24

SEGMENTS	OLD	REGENERATED				
Date	July 22	July 29	August 2	August 5	August 8	August 11
Series A. Worms decapitated July 22.	18	3	3	3	3	4
	18	3	4	4	4	4
	18	3	4	4	4	5
	22	3	4	4	5	6
	24	3	4	5	5	6
	24	3	5	5	6	6
	24	3	5	5	6	7
	28	3	5	6	7	7
	28	4	6	6	7	
	30	4	6	6		
Series B. Worms decapitated July 29.	25	3	3	4	4	4
	25	3	4	4	5	5
	26	3	4	4	6	6
	26	3	4	5	6	6
	28	3	4	5	6	7
	29	3	5	6	7	7
	29	3	5	6	7	7
	29	4	5	6	7	
	30	4	6	7		
	30	4	6	7		
	34	4	6			
	36	4	6			
Series C. Worms with heads intact.	19	3	4	4	4	4
	24	3	4	4	4	5
	24	3	4	4	4	5
	25	3	4	4	4	5
	26	3	5	5	5	5
	26	3	5	5	5	6
	27	3	5	6	6	7
	27	4	6	6	7	7
	29	4	6	6	7	7
	30	4	6	7	7	8
	31	4	6	7	7	9
	33	4	6	7	8	

decapitation upon posterior regeneration from the very beginning of the process. As will be seen from table 5, in which are given the results of this experiment, the number of worms proliferating new tissue two days after the operation (August 6) is twice as great in the control as in the case of the decapitated worms, being 82 per cent of the former and only 42 per cent of the latter. Four days after the operation (August 8) the detrimental influence of the extra operation upon the regeneration of the tail is still apparent, but after a week's time the difference between control and decapitated worms disappears almost entirely.

To sum up the results of the above investigation, it may be said that the additional mutilation of the head in Podarke obscura causes a depressing effect upon the tail regeneration, which is expressed either in a smaller number of regenerated segments or in a greater frequency of regenerated tails with few segments. The effect, however, wears off as the time of regeneration is protracted, and is the more pronounced the more anterior the level, *i.e.* the shorter the moiety of the worm from which regeneration

TABLE 5

August 4—September 2

CONDITION OF WORM	DECAPITATED						HEADS INTACT					
SEGMENTS	REGENERATED						REGENERATED					
Date	Aug. 6	Aug. 8	Aug. 10	Aug. 12	Aug. 14	Aug. 18	Aug. 6	Aug. 8	Aug. 10	Aug. 12	Aug. 14	Aug. 18
1		2 (?)	2	3	3	3			2	2	3	3	3
2		2	3	3	3	3		2	3	4	4	4
3	42% of operated worms commenced to regenerate.	2	3	4	4	4	82% of operated worms commenced to regenerate.	3	4	4	4	4
4		bud	3	4	4	4		2	4	4	4	4
5		bud	3	4	4	4		3	4	5	5	5
6		3	4	4	4	4		2	4	5	5	5
7		bud	4	4	4	5		2	4	5	5	5
8		bud	4	4	5	5		bud	4	5	5	5
9		bud	4	4	5	5		3	4	5	5	5
10		2	4	5	5	6		3	4	5	5	6
11		2	4	5	5	6		3	4	6	7	8
12		2	4	5	5	6						
13		2										

proceeds. The detrimental influence of the extra injury is specially strong during the first few days following the operation, owing probably to the fact that a greater claim is then made upon the organism's reserve formative energy; but if the new tail has already got a sufficient start in regeneration its further progress can not be impeded by an additional mutilation of the organism.

B. RELATION BETWEEN FREQUENCY OF INJURY AND RATE OF REGENERATION

Next in importance to the problem of the relation between the rate of regeneration and degree of injury stands the problem of its relation to the frequency of injury. In earlier studies (Morgulis, '08) I have shown that the regenerative rate decreases with each repeated operation. Similar conditions were also observed in Podarke ('09b), although it was there pointed out that the worms tend to regain their original rate of regeneration. The inference of real importance to be drawn from those experiments is that within a given period of time an organism generates more tissue the more often it has been operated upon. In 1908 Zeleny suggested that after successive operations the rate of regeneration increases, but his data at that time were quite inconclusive. Lately ('09a) he has brought forth additional evidence, obtained with great precaution against any possibility of error, and that investigation has led him to formulate his opinion more carefully, as follows: "The data as a whole make it highly probable that the pure effect of successive removal is either no change in rate of regeneration or an increase in rate" ('09a, p. 508)

I performed a few experiments on Podarke to verify my former observation that the rate of regeneration decreases after repeated mutilations, and in what follows I shall present the outcome of those experiments. It should be recalled that in experimenting with worms one does not encounter such perplexing difficulties in the way of controlling conditions as the crustacea, for instance, offer. Other factors, too, are easily controllable. The experiment consisted simply in allowing some worms to regenerate continuously for several weeks, while on other worms from the

same lot the operation was executed twice during a similar period of time. On looking through table 6, it will be noticed that in the course of four weeks the worms have regenerated on the average 8.4 segments (5 to 14 segments). From previous studies on Podarke (Morgulis, '09b), it is well known that the largest number of segments was regenerated within the first two weeks after the operation.

TABLE 6

JULY 2—JULY 30	NUMBER OF SEGMENTS										AVERAGE
Old segments............	20	17	16	17	17	16	15	15	16	20	18	17
New segments..........	11	5	7	7	8	9	8	14	7	8	8	8.4

Table 7 contains the records of an experiment where the worms were operated on twice during the four weeks, the second operation having been executed at the end of two weeks. At that time the average number of regenerated segments was 6.2, the number ranging from 5 to 8 segments in different individuals. The stage of greatest regenerative rate had thus been passed at the time the regenerated tails were cut off, and the worms were left to regenerate anew for another two weeks. The average number of segments regenerated for the second period of two weeks is only 5.4, ranging from 4 to 7 segments, showing very definitely that the rate of regeneration following the second operation was slower. There is no significance in arguing that the decrease in the rate of regeneration following an operation once repeated is no decrease at all, but the outcome of a "general decline of the physiological

TABLE 7

JULY 2—JULY 16	NUMBER OF SEGMENTS														AVERAGE
Old segments.........	18	21	19	18	18	17	18	17	16	16	14	18	19	17	14	17.3
New segments........	8	5	6	7	7	5	7	6	6	5	7	7	6	6	5	6.2
JULY 16—JULY 30	REGENERATED SEGEMENTS REMOVED JULY 16														
Old segments.........	16	18	18	15	14	16	16	15	13	19	15	dead				16
New segments........	4	7	7	7	7	4	5	4	4	6	4					5.4

activity of the organism," for what else can this fact signify except that the physiological activities are lessened? The argumentation might justly and with equal pertinency be turned against the opposite proposition, viz., that an increase of the regenerative rate after successive injuries is the result of an acceleration of the physiological activities; but nothing could be gained by such an argument, which simply translates a tangible fact into an elusive conjecture, and in neither case leads anywhere.

The fact which commands our attention is that, while the regenerative process after the first operation is already declining, a new operation will cause a new output of regenerative energy exceeding the possible output where there had been no other injury in the meantime. In fact the worms operated on twice at intervals of two weeks regenerated during the space of four weeks an average of 11.6 segments, whereas after a single operation, but within a similar period of four weeks, only 8.4 segments regenerated. In

TABLE 8

JULY 2—JULY 23	NUMBER OF SEGMENTS													AVERAGE
Old segments...........	17	18	15	16	16	18	16	17	16	16	14	16	17	19	16.5
New segments...........	11	9	7	7	8	7	7	5	6	10	7	5	9	9	7.6

JULY 23—AUGUST 13	REGENERATED SEGMENTS REMOVED JULY 23							
Old segments...........	15	17	16	15	15	14	dead	15.3
New segments...........	9	7	6	5	5	5		6.1

other words, there was an average excess of over 3 segments caused by the repeated operation, even though the rate of regeneration the second time was somewhat decreased.

From table 8 it will likewise be seen that, while in three weeks the worms regenerated an average of 7.6 segments (5 to 11 segments), the average number of regenerated segments for three weeks following a repeated mutilation decreased to 6.1 (5 to 9 segments).

C. RELATION BETWEEN SEX AND RATE OF REGENERATION

In studying the rate of regeneration, it is urgent to guard against several sources of possible error, since the conclusions are based almost entirely upon comparisons. In dealing with worms one is doubtless spared many pit-falls arising from the discontinuous method of growth or progressive developmental changes in the experimental animals. Temperature conditions, as well as the factor of size (age?) of the animal and of the level of the cut, can be regulated without much difficulty, and due weight has been given to all these matters in the previous experiments with Podarke. There was, however, one factor still uncontrolled, viz., the influence of the sex of the animal upon its regenerative rate. Generally it is not an easy matter to determine the sex, but I

TABLE 9

SEX OF WORM	MALE			FEMALE		
SEGMENTS	OLD	REGENERATED		OLD	REGENERATED	
Date	July 7	July 21	July 26	July 7	July 21	July 26
1	17	6	5	20	8	5
2	16	7	5	17	7	6
3	19	6	5	20	8	6
4	19	9	6	19	6	6
5	15	4	6	18	8	7
6	16	5	7	17	7	7
7	18	7	7	15	8	7
8	16	6	7	18	8	7
9	16	4	7	16	6	7
10	18	5	7	16	7	7
11	16	7	7	17	5	7
12	18	8	8	17	9	8
13	15	9	8	19	9	8
14	17	9	9	17	7	9
15	18	8	9	14	7	9
16	17	4	10	16	6	9
17	16	8	10	20	6	10
18	18	6	10	14	6	10
19	15	7		19	8	10
20	19	7		19	6	10
21				18	4	

have availed myself of an opportunity to investigate this factor when a number of specimens were found at the breeding season whose sex could readily be distinguished by the presence of eggs or of sperms within the body. Twenty worms of each sex were cut in two about the middle of the body, and left to regenerate new tails. As will be seen from table 9, both the males and the females regenerated from 4 to 9 segments in two weeks, and from 5 to 10 segments in twenty days. It is quite evident from this that the sex of the animal has no influence upon its regenerative power, and this factor, though not considered in earlier experiments ('09b), probably had no effect upon the results.

D. RELATION OF THE AMOUNT OF REMOVED TO THE AMOUNT OF REGENERATED TISSUE

In a previous publication (Morgulis '09b) it was maintained that in Podarke the regenerated tail does not reach its original length when it grows from about the middle of the body, although the full number of lost segments is usually restored when only a small part of the tail has been detached. About the same time a paper was published (Ellis, '09) in which this matter of the relation between the amount of removed and of regenerated tissue was treated more fully, the results leading to a similar conclusion, that "regeneration ceases before the part removed has been completely regenerated" (p. 444).

Since I stated the matter at first more as an opinion than as a fact, I have now undertaken to verify the conclusion by a special experiment in which record has been kept of both the removed

TABLE 10

JULY 6—AUGUST 28		NUMBER OF SEGMENTS															AVERAGE
Removed......	20	20	24	24	24	26	26	26	26	28	28	29	29	30	30	31	32	27
Regenerated { August 6.	7	7	8	8	8	8	8	8	9	9	9	10	10	10	11	12	14	9.2
August 28.	8	9	10	16	9	9	10	11	9	12	8							11.0

and the regenerated segments. The results of examinations made 31 and 53 days after the operation are given in table 10. The average number of removed segments was 27, or a little over one-half of the worm's body. The first examination of the regenerated tails showed that on the average after 31 days only 9.2 segments, or practically one-third of the number lost had regenerated. Fifty-three days after the operation 11 segments on the average (8 to 16 segments) had regenerated, or about 0.4 of the amount removed. The worms were not examined at later periods, but with the knowledge we now possess concerning the phases of regeneration, it may be said with certainty that the regenerative process had already reached practically a standstill, and that very few segments were added subsequently.

The shortening of the tail thus effected by regeneration does not, however, destroy the proportions of the worm, as the whole organism apparently undergoes a corresponding reduction in dimensions. The result of the regeneration of about one-half of the animal is, therefore, to produce smaller worms, but such as are otherwise perfectly normal.

CONCLUSIONS

Among numerous perplexing problems with which it is the biologist's lot to deal, the cessation of the growth of an organism when it has attained a certain form is a matter of no small difficulty to understand. Attempts to explain the phenomenon on mechanical principles are conspicuously inadequate. Investigations on regeneration show that, whereas growth may already have been brought to its normal termination, the capacity to grow is still unabated, and that there is sufficient potentiality in reserve to make good a substantial part of any lost portion. Of course, even formative growth on the part of an organism is never reduced to absolute zero, and while the organism maintains itself at a more or less definite *status quo*, separate parts or organs, as for instance the skin, may still retain the power to grow.

Experiments have proven conclusively that regeneration may be repeated several, and in some cases even many, times in suc-

cession, an enormous amount of growth energy being thus uti-
lized, which would otherwise have remained dormant in the organ-
ism. The evidence of economy with which this inherent power
of growth of the organism is used to compensate for a mutila-
tion is a problem which we cannot go into at this moment. The
interesting facts brought out by most of the recent researches
are, that the lost portion is not fully restored, and, as I have also
observed in Podarke, that regeneration produces smaller individ-
uals but such as have normal proportions, the new growth being
apparently brought to a termination when a definite form has
been established. It should be emphasized here that the cessa-
tion of regenerative growth does not imply an exhaustion of the
regenerative energy, for, as experiments on regeneration after
successive injuries show, a repeated stimulation will overcome
the inertia of the organism and set its formative forces into activ-
ity once more. Whether or not a repetition of the injury causes
an acceleration of the regenerative process—and apparently
either condition may exist—it causes an additional output of
regenerative energy, and the quantity of tissue generated after
several operations greatly exceeds that produced after a single
operation. Likewise, a greater degree of injury—whether increas-
ing or leaving unaffected the regenerative power or even decreas-
ing it, as in the case of Podarke—results in a larger output of
regenerative energy.

The old problem of the cessation of growth recurs under a new
aspect: Why does the regeneration of an organism cease? And
why does it cease before the original size relations have been
restored, notwithstanding the reserve potentiality to further re-
generation? These are questions which must still await a solution.

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EMBRYOLOGICAL STUDIES WITH THE CENTRIFUGE

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THIRTEEN FIGURES

The following experiments with Cyclops fimbriatus were begun at Columbia University in the spring of 1908 and those with Arbacia, at Wood's Hole in the summer of 1909. In respect to stratification of materials, direction of cleavage and development of normal embryos from centrifuged eggs Cyclops gave essentially the same results as others had obtained with Arbacia. The materials of the centrifuged eggs are separated into three layers, oil, protoplasm and yolk. Normal embryos develop from the eggs, whether centrifuged at the stage when the segmentation nucleus is present or after the cleavage spindle has formed. The eggs do not orient themselves in the machine, nevertheless the first cleavage is always perpendicular to the induced stratification. An examination showed that the segmentation nucleus is easily driven into the protoplasmic band and that the cleavage spindle may also be driven into the protoplasmic layer at any stage in its development but it takes a stronger force to displace the spindle than to drive the segmentation nucleus. The materials of the egg also are harder to separate by centrifuging as the time of first cleavage approaches. There is apparently some increased tension in the egg substance but the karyokinetic figure is not so rigid as to be prevented from its normal functioning by the rearrangement of the materials of the egg.

Some light was thrown on the structure of the karyokinetic figure by the experiments upon Cyclops. The chromosomes, spindle fibers and the centers of the asters together comprise a unit system which bends, but is not torn apart, by the passage

of the large yolk spheres around it.   All appearance of astral rays
is lost after centrifuging.   Nothing remains but a disc of basic
granules, marking the center of the aster.   This shows that in
Cyclops the astral rays are not fibers.   The spindle "fibers," in
contrast to the astral rays, not only have the appearance of con-
tinuous threads but behave as such, being in some cases bent.   A
separation of the materials composing the center of the aster
results from centrifuging.   In the normal egg the aster is composed
apparently only of acid substance, but after centrifuging its
reaction is basic, showing that in the normal egg the basic is also
present together with the acid substance.

The cleavage spindle of Arbacia may also be pushed out of its
normal position as a result of centrifuging.   Its subsequent appear-
ance is in every way normal and the cleavages that follow are
normal.   In eggs killed immediately after centrifuging the astral
rays extend radially into the protoplasm and are perfectly straight
as in the normal egg, despite the rearrangement of materials.
This seems to be opposed to a theory which supposes the astral
rays in the sea-urchin to be true fibers since it is hard to conceive
of a radial system of fibers remaining undistorted under such
conditions.

In the remaining experiments made with Arbacia several points
were taken up which had not been sufficiently studied in previous
work.   A detailed study was made of the percentage of normal
embryos developing from eggs centrifuged at different stages of
development and at various speeds.   The few cases in which the
first cleavage after centrifuging was parallel to the layers instead
of perpendicular were examined.   This type of cleavage occurs
only in Arbacia eggs which are fertilized after centrifuging.   The
sperm pronucleus, being unaffected by the force, may approach
the egg pronucleus from any direction with reference to the lay-
ers, hence in some cases its direction is such as to cause the spindle
to form with its axis at right angles to the layers.   The relation
of the first cleavage plane to the micropyle pole was studied in
Toxopneustes by Boveri who described it as passing always
through the micropyle pole.   In Arbacia I found that while this
relation generally holds good there are some eggs which vary
widely from the rule.

I wish, here, to acknowledge my indebtedness to Dr. T. H. Morgan, under whose direction the work was done.

EXPERIMENTS WITH CYCLOPS FIMBRIATUS

The normal development

Cyclops fimbriatus, one of the smaller species of cyclops is easily kept alive in manure cultures, containing protozoa, where it reproduces rapidly in the late winter and early spring. The rate of reproduction at any time of year, however, is influenced by the amount of food material in the culture.

The egg sacs of the species are comparatively small, containing five to ten opaque blue-gray eggs. Each egg is enclosed in a separate compartment within the sac. The transparent walls separating the eggs are particularly well seen in old sacs from which the nauplii have just escaped. In focusing with the microscope on a young egg sac the walls are also visible and are sometimes easily confused with the first cleavage plane. The eggs are fertilized in the oviduct of the female. Nauplii hatch from the sac seven or eight days after fertilization, and reach the adult stage and produce egg sacs two weeks later. There is no evidence that the eggs ever develop parthenogenetically. To test the point, however, I isolated the nauplii of a sac when they were only two or three days old and kept them isolated for four weeks. The females did not produce egg sacs during that time, which was two weeks beyond the normal time of maturity. When males were added, egg sacs were produced in the course of two or three days. The development of the eggs is not dependent upon the attachment of the sac to the animal body, for normal nauplii hatch quite as quickly when the sacs are removed and placed in tap water.[1]

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[1] In order to study the living material microscopically the cover slip should be supported just enough to avoid crushing the animal and yet keep it from moving about. For preserving the eggs Carnoy's mixture is an excellent fixative. The adult animals are better preserved with hot sublimate acetic. For a great deal of the work it was necessary to embed isolated egg sacs and for this purpose I adopted the method of wrapping the sac in a small piece of very thin salamander epithelium. The egg sac was first stained for several hours in a weak solution of orange

Sections of normal eggs which have just been deposited in the
sacs contain the second polar spindle (fig. 1).   The spindle is
small and lies embedded in a disc of purple staining granules at
the periphery of the egg.   The rest of the egg is filled with the
characteristic large yolk spheres, which are separated from each
other by a film of purple granules like those surrounding the
spindle.   The purple granules occupy the areas which in living
eggs are clear protoplasm.   This has been seen in centrifuged eggs.
Whether these "granules" are real granular inclusions in the more
fluid protoplasm or simply coagulation products is an open ques-
tion.   If they are coagulation products only, then their position
in the section probably represents the extent of the protoplasm
in the centrifuged egg.   But if they are true granular inclusions
it may well be that they are to some extent separated from the
surrounding protoplasm and consequently that the entire proto-
plasmic portion of the egg is not indicated by the position of these
granules.   The evidence, discussed later on, pointing to a "ground
substance" which is undisturbed by the centrifuge, concurs with
the latter idea.

The polar spindles which I found (five in number) were all
late anaphases (fig. 6) with no evidence of astral rays at either
pole.   Half an hour after the eggs have been deposited in the sac
the segmentation nucleus lies in the center of the cell and is much
enlarged.   The two pronuclei that compose the segmentation
nucleus, however, have not fused but lie flattened against each
other.   Haecker has described gonomery in another species of

G in 95 per cent alcohol, to make it more plainly visible and then wrapped in
a piece of epithelium from which as much water as possible had been drained.
Care must be taken to avoid air bubbles inside the bundle in the process of wrap-
ping, else the sections will be torn to pieces in cutting.   It is also well to let the
epithelial ball dry off somewhat on the outside before replacing in 95 per cent alco-
hol in order to stick the folds together and prevent unwrapping and loss of mate-
rial in the paraffin.   The eggs were imbedded in fifty-eight paraffin and sections
cut 3 μ in thickness.   The method proved very successful not only with single egg
sacs but also with groups of sacs and even adult animals.

Delafield's haematoxylin followed by orange G has been used for practically
all the staining.   I tried iron haematoxylin counterstained with orange G.   While
it offers an interesting contrast in some cases to the Delafield stain, it does not
bring out very well the relation of the centrifuged materials.

cyclops, where he believes it persists to a very late stage in development. That it persists in the spindles is shown by the frequent separation of the two parts of the spindle during centrifuging (fig. 3). The asters at the poles of this double nucleus consist of masses of protoplasm, indistinguishable from that which separates the yolk spheres. There is no visible centrosome or appearance of astral fibers in the protoplasmic mass. The only radial appearance is afforded by the yolk spheres, which radiate from this center in rather regular rows, and by a few paths of protoplasm, broader than the rest, passing out from the central mass between the rows of yolk spheres (fig. 7).

The earlier nuclear changes of living eggs are readily followed with the lower powers of the compound microscope. Eggs which have just been deposited in the sacs have a light gray spot near their periphery; this is the second polar spindle. Since there is no definite relation between the position of the spot and the axis of the egg sac, it is probable that the eggs are not oriented in the sac. At the end of half an hour the second polar body has been given off, the nucleus has moved to the center of the cell, is much enlarged and is a distinct gray disc. Later it seems to fade out a little, gradually elongates in the long diameter of the cell, and the outline of spindle and asters can be seen. With the oil immersion lens the rows of yolk spheres, radiating from the gray center of the aster, may also be seen.

The first cleavage plane appears an hour after the egg sacs have formed. The daughter nuclei round off into gray discs in the center of the blue-gray cells, and in ten to fifteen minutes begin to elongate in a direction parallel to the first cleavage plane. The second cleavage follows in half an hour after the first, and the third cleavage is complete at the end of the second hour. The egg then consists of eight equal cells. Beyond this point the cleavages have not been traced. The eggs are crowded together in the sac so closely that most of them are flattened in one diameter. Indeed, the egg at the open end of the sac is the only one which is spherical. I examined between fifty and sixty normal eggs and found the cleavage plane regularly cutting through the shortest diameter of the cell. McClendon has found that in certain para-

sitic copepods, also, the direction of the first cleavage plane is influenced by compression of the eggs in the egg strings as well as when the eggs are compressed by artificial means.

Development of the egg after centrifuging

For the following experiments a hand centrifuge was used, and the animals were revolved for $1\frac{1}{2}$ minutes (9000 rev.) at a radius of 7 cm. The adult animals, though thrown to the bottom of the tube and held there, recover in two or three seconds and are apparently uninjured.

The materials of unsegmented eggs, which have been centrifuged for $1\frac{1}{2}$ minutes (9000 rev.) at a radius of 7 cm. are divided into three layers (fig. 8). At the centripetal pole is a cap which is white by reflected light under the microscope and gray-green by transmitted light. The rest of the centripetal hemisphere contains a clear band of protoplasm, and the centrifugal pole is blue-gray in appearance because of the densely packed yolk spheres. The white cap turns black with osmic and is partially dissolved in alcohol. Hence it is probably chiefly oil as in other eggs.

A centrifuged egg, sectioned and stained with Delafield's haematoxylin and orange G, is shown in fig. 2. The yolk spheres, crowded together in one hemisphere, take the yellow stain. In a well centrifuged egg there are no purple granules between the yolk spheres as there are normally. The rest of the egg with the exception of the aster stains with haematoxylin. The asters are yellow. The green cap is only distinguishable by the vacuoles left where the oil has been dissolved out in the alcohol. The mitotic figure, whether it be resting nucleus or cleavage spindle, lies in the purple hemisphere near the cap and, in the case of the spindle, parallel to the cap.

When a living egg has been centrifuged (just after the segmentation nucleus has enlarged), and the separation into layers is very distinct, movement is visible among the small green spheres of the cap. At first it is not different from Brownian movement. But as the spindle forms, clusters of the spheres sway in one direction or another, indicating the flowing movements in the sur-

rounding protoplasm. Gradually the green spheres move along the periphery, forming two points on opposite sides of the egg, through which the first cleavage plane always cuts. During the same interval the yolk spheres along the protoplasmic border show a vibratory sort of movement and slowly encroach on the clear band, the movement into the protoplasm being most marked opposite the points of the green cap. After ten to fifteen minutes the clear band is quite blurred so that the nucleus or spindle, as the case may be, is roughly outlined as in the normal egg and on the yolk side of the aster the radial arrangement of the yolk spheres is often quite clearly seen. The first cleavage is always perpendicular to the layers so that even in eggs which have been centrifuged in the anaphase or telophase the process of remixing goes on unhindered for another half hour, or in many cases an hour, till the first parallel cleavage occurs. That the tendency of the centrifugal layers to remix is not responsible for the normal development after centrifuging is shown by an experiment in which the unsegmented eggs were placed in the water centrifuge and revolved at a moderate rate of speed for 3 hours. The separation of materials was thus maintained until after the third, or equatorial cleavage had come in. Thus four cells contained the yolk spheres and the other four cells contained protoplasm and oil. Normal embryos were obtained from such eggs. Therefore, the separation of the visible substances does not in this case lead to abnormality.

The direction of the layers is the same in all the eggs of a given egg sac. The sacs are free to swing in any direction, being attached to the animal only at one point. Nevertheless the direction of stratification with reference to the axis of the sac is rarely the same in the two egg sacs of a given individual. The egg sac as a whole, therefore, does not orient itself to the direction of the centrifugal force. The question of the orientation of the individual egg is more difficult to determine and could not be proved from the unsegmented egg. It is possible, however, to show that immediately after the first cleavage, the eggs do not orient themselves. For, if such eggs are centrifuged the direction of the layers

is the same in all the eggs of the sac; but, owing to the variant direction of the cleavage planes, the layers may be parallel, perpendicular or oblique to the cleavage (fig. 10). All three conditions may occur in the same egg sac. So far as visible substances are concerned, there is more reason to expect orientation in the two-cell stage than before. On the other hand, if the egg is supposed to orient itself with respect to its original egg axis or polarity, this factor should be as potent after cleavage as before. I think, therefore, it is fair to assume that the eggs do not orient themselves in the machine.

In spite of such disturbances of the egg normal embryos regularly result and reach the adult stage at the end of three weeks. Egg sacs of animals, which have hatched from centrifuged eggs, have been centrifuged and normal embryos obtained. Moreover, normal embryos result no matter whether the egg is centrifuged while the resting nucleus is intact or after it has broken down and the spindle is formed. The proof of this point was made possible by the fact that the eggs in both sacs of a single animal are in approximately the same stage of development. Their cleavages cut through within three or four minutes of each other. Thus it was possible, by killing one of the sacs immediately after centrifuging, to determine the stage at which the eggs were centrifuged. After the cleavage of the second sac had been observed the sac was isolated in a watch glass, containing tap water to which a few drops of culture media were added from a jar in which there had been no cyclops. It may be said here that in all isolation of centrifuged eggs care was taken to guard against the possible introduction of egg sacs or small nauplii from other individuals either by way of pippettes or culture media.

The spindle after centrifuging

Preserved and sectioned sacs from four animals contained eggs with metaphase spindles. In the corresponding living sacs the eggs divided perpendicularly to the layers in three to ten minutes after centrifuging and normal embryos hatched in a

week and produced normal adults. In one case it is certain that a nauplius hatched from every egg in the sac. In the other cases a majority of the eggs hatched. Two sacs which were known to have been centrifuged before the nuclear walls had broken down also gave rise to normal embryos. The cleavage planes and hence the spindles in the eggs of a normal sac, as already pointed out, bear no constant relation to each other, though they are to a certain degree determined by pressure conditions in the sac. Centrifugal force acting upon the sac, therefore, acts upon the spindle of each egg from a more or less different angle. Moreover, in each of the four egg sacs described above the layers bore a different relation to the axis of the sac. These considerations, together with the fact that even in normal sacs all the eggs do not develop, make it probable, I think, that the spindle is never permanently injured by centrifuging.

The relation of the nucleus and of cleavage spindles to the layers is the same in eggs centrifuged after, as in those centrifuged before cleavage. When the stratification is either parallel or perpendicular to the cleavage the long axis of the spindle is always parallel to the layers and also to the first cleavage plane. When the stratification is oblique to the cleavage the spindles conform to the stratification and make an acute angle with the first cleavage plane (fig. 5). Moreover, in the oblique eggs the spindles of the two blastomeres lie sometimes at right angles to each other, whereas normally they are parallel (fig. 4).

The condition of the spindle after centrifuging varies and the variation affords one of the most striking evidences of spindle movement. The sectioned sac shown in fig. 3 illustrates the different conditions. Egg (a) represents the same condition as that found in the sea urchin. The spindle lies close under the white cap and its parts retain their normal relation to each other. In (b) and (c) the equatorial portion of the spindle lies close under the cap but the poles are bent far down toward the yolk layer.

That the distortion of these spindles is not to be explained by differences in specific gravity of its parts is shown by the straight spindle in (a). It is rather due to the shape of the cell. Egg (a),

lying at the end of the sac, is nearly spherical and the action of the force has been such as to broaden the cell in the direction of the layers. There is, therefore, plenty of room for the spindle to lie straight. In (b) and (c), however, the already elongate and flattened eggs were, if anything, more elongated and consequently flattened by centrifuging and the spindle has been forced into a position with its axis in the shortest diameter of the cell. Since this diameter is shorter than the normal length of the spindle, the spindle must bend.

Egg (c), in which one aster lies nearer the yolk than the other, suggests what the normal position of these spindles may have been, for their position in the short axis of the cell is proof that there has been more than a simple shifting of the karyokinetic figure to one side. The egg is so much flattened that the diameter of the egg perpendicular to the plane of the section is probably as long as the diameter perpendicular to the stratification. If the spindle had lain normally in the former position the natural result of centrifuging would have been a simple shifting to one side of the spindle, in which case an equatorial plate would have shown in the section instead of the longitudinal view of the spindle. If, however, its original position were perpendicular to the layers, it would shift as a whole to the centripetal pole. The yolk spheres in their passage would drag the centripetal aster back, thereby bending the spindle. The pressure of the yolk spheres being greater in the short than in the long diameter of the cell would cause the aster to be dragged in that direction and account for the spindle's abnormal position in the short diameter of the cell. Such a condition in process is shown in fig. 1, where the egg was not centrifuged completely as shown by the fact that the yolk spheres are not driven completely to the centrifugal pole. The spindle is only partially bent and one pole is still embedded in yolk. In fig. 3 (c) the process is nearly complete. One pole, however, is still nearer the yolk layer than the other. With longer centrifuging the asters would, probably, assume a symmetrical position with regard to the layers.

The structure of the aster

This brings us to a consideration of the asters themselves. The normal aster (fig. 7) as represented in section consists of a mass of purple staining granules, continuous with and indistinguishable from the granules separating the yolk spheres. Even the highest magnification fails to reveal any trace of centrosome or astral fibers. There is no radial arrangement apparent in the central mass. The surrounding yolk granules, however, are arranged in quite regular rows, radiating from the purple area and between some of these rows the paths of granules are wider than elsewhere, giving an appearance of rays. But these rays appear to be entirely granular and not to contain fibers.

The partially centrifuged egg (fig. 1) indicates that there is something stable in the structure of the granule mass, which represents the aster, for the yolk spheres do not move into it, but maintain to some extent their radial arrangement. An egg in which the substances have been well separated (fig. 3) illustrates one of the most peculiar things in the behavior of the aster. Whereas in the normal egg the aster is purple, in the centrifuged egg the aster, lying in the purple hemisphere, is differentiated from the surrounding granules by its yellow stain. It is an irregular disc of yellow granules whose edges fade into the purple. I have tried to demonstrate the presence of fibers radiating from the center of the disc by using orange G, Congo red or eosin without any counterstain. But the stains have failed to show any suggestion of an astral fiber. The spindle fibers, on the other hand, are visible even with these simple stains though they are not very clear unless haematoxylin is used. In some preparations the aster is homogeneous, in others there is a more or less distinct radial arrangement of the granules with clear spaces between. The latter is, I think, due to poor preservation.

The same change in staining capacity of the aster is found in eggs centrifuged just before the nuclear walls have broken down. Hence the affinity for the acid dye is not due to the age of the aster. There are present in the normal aster both basic and acid

granules. The acid granules stain with haematoxylin and are
so dark as to obscure the basic granules. When, however, the
egg is centrifuged, the acid granules are driven out of the struc-
ture, which shows that their specific gravity is greater than that
of the basic granules, else no separation would occur. Whether
or not the acid granules vary among themselves in specific grav-
ity, is by no means proven. It is true that in a rounded egg (fig.
3 a) the spindle and asters lie close under the cap. Nevertheless,
there are purple granules to either side of the asters and also
between the asters and the centripetal pole, for they extend into
the region of the cap. This seems to show that the purple granules
are of varying specific gravities, ranging from granules heavier
than the basic ones to those lighter. If such a hypothesis is
true, there should be some purple granules persisting among the
yellow ones in the aster and the sections seem to bear this out,
though the evidence is not clear enough to be conclusive. The
other possibility is that the acid granules are all heavier than the
basic ones but that the centrifugal force has not been sufficiently
strong to drive them all down. This explanation has the merit
of simplicity and if it be true, the disc of basic granules is shown
to be a comparatively stable structure for it does not become
flattened out against the oil cap, but maintains its rounded shape
and its position at the poles of the spindle.

In view of the separation of granules in the aster the stain-
ing reaction of the eggs in the oviduct is suggestive. The nucleus
is surrounded by a ring of protoplasm which stains with haema-
toxylin. In order to show well the contents of the nucleus at this
period it is necessary to stain a comparatively short time, since
the chromatin takes up the stain very rapidly. As a result the
surrounding rim of protoplasm was not so deeply stained as in
the older material and some yellow granules could be seen among
the purple ones. This protoplasmic ring forms the mass sur-
rounding the second polar body (fig. 6) and subsequently gives
rise to the asters.

Evidence for a substance unaffected by centrifuging

When the egg is centrifuged the yolk spheres are driven into one hemisphere with no visible substance between them. Nevertheless the fact that, when so driven, the globules maintain their spherical form and neither resume the appearance which they have in the yolk glands of the parent nor resemble the mass of yolk granules set free when the egg is crushed, indicates that the clear spaces between the globules in the yolk hemisphere of the centrifuged egg contains an unstained ground substance of some sort, which is not separated by centrifuging. In partially centrifuged eggs a few purple granules still remain in the spaces between the yolk globules. Moreover, without admitting the presence of some substance other than yolk in the centrifugal hemisphere we could not account for the normal division, since a concentration of yolk in one part of an egg usually interferes with or even inhibits cleavage in that part. Cyclops, with its large yolk spheres, has shown better than any other eggs that the purple granules may be entirely pushed out of the centrifugal hemisphere, leaving colorless spaces between the globules of yolk; and that these spaces contain enough of the essential protoplasm to make normal cell division possible. If a colorless ground substance does exist we should expect to find that the acid and basic "granules" are really granular inclusions and not artifacts.

The frog's egg when centrifuged at about the same speed as that used for cyclops fails to segment in the yolk hemisphere. The frog's egg, however, has its yolk largely in one hemisphere in the normal condition, so that the result of a given speed of the centrifuge is probably equal to the effect of a much stronger force applied to cyclops or sea urchin eggs where the materials are uniformly distributed. If we centrifuged cyclops or the sea urchin sufficiently hard I believe it very likely that we could drive the yolk so completely to the centrifugal pole that the ground substance would be entirely pushed out from between the globules and a result comparable to that in the frog would be obtained. The yolk- and the granule-containing alveoli probably move

through the fluid ground substance and the persistency with which the medium retains its position may be due to a physical tendency to adhere to the yolk spheres or granules.

EXPERIMENTS WITH ARBACIA PUNCTULATA

The object of my work at Wood's Hole during the summer of 1909 was to carry out experiments upon the cleavage stages of arbacia similar to those already described for cyclops. There were also several points which had not been cleared up in the work done by Dr. Morgan and myself at Wood's Hole in 1908.

Mortality after centrifuging

Lyon showed in 1905, that when the egg of arbacia is centrifuged its materials are divided into four layers; an oil cap and protoplasmic band in the centripetal hemisphere and in the other hemisphere a broad band of yolk with the pigment massed at the outer pole. He also obtained a great many normal embryos from such eggs. Detailed experiments had not been made to show the relation of mortality, either to the length of exposure of the eggs to a high centrifugal force or to the stage in its development at which the egg was centrifuged.

Eggs and sperm from a single pair were used throughout one series of experiments in order to eliminate error arising from differences in animals. When six or seven lots of eggs were centrifuged in one series of experiments, the eggs and sperm used for the last lot had necessarily been exposed in the bowl of sea water for thirty or forty minutes, while those in the first set were fresh from the animal. To meet this difficulty when the experiments were repeated the sequence was changed. If in the first series the first lot of eggs were exposed to the force for only a short time, in the next series the first lot were exposed for a long time. The counts from several series of experiments were combined.

The speed of the centrifuge was as nearly constant throughout the experiments as was possible with a hand centrifuge, varying from 6000 to 7000 revolutions a minute. In making the counts

of the 16-cell stage several microscopic fields were picked out at random in each watch glass and all the normal and abnormal eggs in each field were counted. Wherever the figures are given for gastrula stages, they are necessarily less accurate because it is difficult to compare the number of swimming gastrulas scattered around the edges of the dish with the dead eggs concentrated at the bottom. However, I counted always an equal number of the most crowded fields, both of dead and living, and in this way endeavored to obtain ratios which could be compared. The error in the individual cases gives too high a per cent of mortality.

TABLE 1

STAGE AND DURATION OF EXPOSURE IN THE CENTRIFUGE	16-CELL STAGE		PER CENT MORTALITY
	Normal	Dead	
Normal control—fertilized immediately after removal from the animal....................................	259	123	32
Centrifuged for 3 minutes—10-15 minutes after removal from the animal—then fertilized....................	186	67	26
Centrifuged for 2 minutes—20-30 minutes after removal from the animal—then fertilized....................	195	17	8
Centrifuged for 1 minute—40-50 minutes after removal from the animal—then fertilized....................	204	69	25
Centrifuged for 2½ minutes—45 minutes after fertilization...	207	51	19
Centrifuged for 2½ minutes—50 minutes after fertilization...	141	26	15

The results of the experiments are given in the accompanying tables. All the eggs used in the experiments of table 1 were taken from a single pair of animals. A comparison of the percentages of mortality in the table indicates that at least an hour's exposure in the dishes before the beginning of the experiment does not injure the eggs or sperm, for the mortality is greater in the controls than in the eggs which have stood thirty and fifty minutes in the dishes. The table also indicates that there is no greater mortality in eggs, which are centrifuged in the spindle stages preceding the first cleavage, than in those centrifuged before fertilization or in the controls.

In table 2 are set down the results of a series of experiments in which the unfertilized eggs of a single female were centrifuged at a high rate of speed, 6000 to 7000 revolutions a minute, during different periods of time, varying from one-half to three minutes. The result of this experiment may be taken to indicate a slight increase in mortality over the controls due to longer periods of centrifuging. The increase, however, is comparatively slight and not perfectly regular. Moreover, when the numbers of normal and abnormal eggs from several experiments are put together as in table 3 no such consistent rise in mortality is apparent.

The evidence, I believe, shows that there is no direct relation between the percentage of mortality and either the duration of the force or the stage in development at which the eggs were centrifuged. The counts from several series of experiments have been put together in table 4, and it is interesting to see that the percentage of mortality among the gastrulas in the centrifuged eggs does not vary greatly from that of the controls. Such variation as does occur is not consistent and may easily be due to error in counting.

Direction of cleavage after centrifuging

In regard to the development of the eggs after centrifuging, Lyon and Morgan found that in the majority of cases the first cleavage was perpendicular to the stratification, while the later gastrula pole bears no definite relation to the layers. By isolating eggs and recording their development to the gastrula stage, I found, 1908, that gastrulation takes place at the micromere pole as in normal eggs and is quite independent of the centrifugal layers. At the same time by use of Boveri's india ink method Morgan showed that the micromeres form approximately opposite the micropyle. This is the relation which Boveri describes for the normal egg of Toxopneustes except that he speaks of the micromeres as being exactly opposite the micropyle. The question of the exactness of the relation became interesting because the cleavages so evidently conform to the stratification of the centrifuged egg. Therefore I studied, last summer, a set of normal eggs in india ink solution and found that the relation between

TABLE 2

STAGE AND DURATION OF EXPOSURE IN THE CENTRIFUGE	16-CELL STAGE		PER CENT MORTALITY
	Normal	Dead	
Normal control—fertilized immediately after removal from the animal..................................	203	20	9
Centrifuged for $\frac{1}{2}$ minute—then fertilized..............	62	2	3
Centrifuged for 1 minute—then fertilized..............	58	10	14
Centrifuged for $1\frac{1}{2}$ minutes—then fertilized............	143	29	16
Centrifuged for 2 minutes—then fertilized.............	80	20	20
Centrifuged for $2\frac{1}{2}$ minutes—then fertilized............	144	33	18
Centrifuged for 3 minutes—then fertilized.............	153	34	18

TABLE 3

Tables 1 and 2 combined

STAGE AND DURATION OF EXPOSURE IN THE CENTRIFUGE	16-CELL STAGE		PER CENT MORTALITY
	Normal	Dead	
Normal control..................................	461	143	23
Centrifuged for $\frac{1}{2}$ minute—then fertilized..............	62	2	3
Centrifuged for 1 minute—then fertilized..............	262	79	23
Centrifuged for $1\frac{1}{2}$ minutes—then fertilized............	143	29	16
Centrifuged for 2 minutes—then fertilized.............	275	37	11
Centrifuged for $2\frac{1}{2}$ minutes—then fertilized............	144	33	18
Centrifuged for 3 minutes—then fertilized.............	339	101	22
Centrifuged for $2\frac{1}{2}$ minutes—45 minutes after fertilization..................................	207	51	19
Centrifuged for $2\frac{1}{2}$ minutes—50 minutes after fertilization..................................	141	26	15

TABLE 4

STAGE AND DURATION OF EXPOSURE IN THE CENTRIFUGE	GASTRULA STAGE		PER CENT MORTALITY
	Normal	Dead	
Normal control..................................	1038	931	47
Centrifuged for 2 minutes—then fertilized.............	572	322	36
Centrifuged for 2 minutes—40 minutes after fertilization..................................	398	348	46
Centrifuged for 2 minutes—50 minutes after fertilization..................................	175	215	55

micromeres and micropyle is only approximate even in the normal
eggs of Arbacia (fig. 9). Boveri, also, describes the first cleavage
as passing always through the micropyle pole. I found one
normal egg in which the first cleavage plane cut through at right
angles to the polar axis.

The eggs which I isolated in 1908, not only show that the
normal relation of micromeres and gastrulation existed in the
centrifuged eggs, but also that the first cleavage plane does not
always pass through the micropyle pole as Boveri describes.
In studying these results, however, attention became centered
on the small, but nevertheless existent, number of eggs in which
the first cleavage was more nearly parallel to the stratification
than perpendicular.

A small number of parallel cleavages had been reported in
all the earlier work in which the eggs were centrifuged before fer-
tilization. But so far as I know no observations had been recorded
on this point in eggs centrifuged after fertilization. My first
experiment, therefore, was to centrifuge two sets of eggs, one
before fertilization, the other forty-five minutes after fertiliza-
tion. In each case the watch glass containing the centrifuged
and fertilized eggs was placed under the microscope and a single
field was observed till the eggs had divided. In a small field of
the eggs, centrifuged before fertilization, only thirteen eggs
divided. In five of those eggs the cleavage was parallel to the
stratification. In a field containing over a hundred eggs, centri-
fuged after fertilization, the cleavage was visible in eighty-eight
eggs. They all divided within twenty minutes after removal from
the centrifuge and in every case the cleavage was perpendicular
to the stratification. This suggested that the irregularity of the
cleavage in the eggs centrifuged before fertilization might be due
to the fact that in those eggs the sperm enters after the centri-
fuging, and the sperm head and the aster are, therefore, not
directly affected by the force; while in eggs centrifuged just before
cleavage the whole karyokinetic figure is affected.

With this possibility in mind I centrifuged a large number of
eggs before fertilization and killed some immediately after removal
from the machine and others at five minute intervals after fer-

tilization to the first cleavage. These eggs have been sectioned and stained with Delafield's haematoxylin and orange G. The yolk hemisphere takes up the orange stain and the protoplasmic band and such part of the cap as has not dissolved out in the alcohols are purple.

The egg pronucleus just after centrifuging lies in the center of the protoplasmic band and its position is apparently unchanged after fertilization. Eggs which were killed ten or fifteen minutes after fertilization show the sperm head, sometimes with its aster.
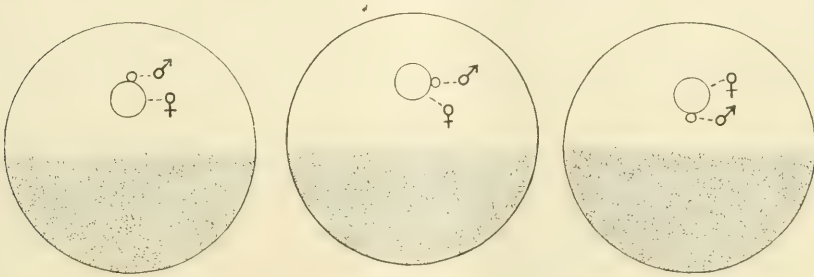
Fig. a

It lies comparatively near the periphery of the egg but bears no relation to the stratification, being found sometimes in the yolk and sometimes in the protoplasm. From this it is evident that the point of entrance of the sperm is not in any way determined by centrifuging.

Twenty minutes after fertilization the male pronucleus is enlarging and fusing with the egg pronucleus (fig. a). As the sperm head may enter from any side of the egg with reference to the stratification so it may fuse with the egg pronucleus on any side. Thus as the centrosomes divide moving to the poles of the nucleus the spindle may form at any angle to the stratification. Fig. 2 shows an egg in which the spindle has formed at right angles to the stratification and from which a parallel cleavage would result. The fact that such a large proportion of the cleavages are perpendicular to the stratification is due to the tendency of the spindle to form in the direction of least resistance. In centrifuged eggs it is very probable that the upper boundary of the

yolk layer, because of its relative density, may have much the same influence upon the karyokinetic figure as does the cell wall separating two blastomeres of the two-cell stage. Spindles, therefore, which start to form at an angle to the stratification, would tend to slip around into a position in the long axis of the protoplasmic layer and consequently parallel to the stratification. The cases of parallel cleavage then would be those in which the male pronucleus approaches the female pronucleus in a direction parallel to the layers; and the centrosomes pass around the nucleus in such a way that the spindle forms directly across the proto-plasmic band perpendicular to the layers and is so symmetrically placed that it cannot slip around into the longer axis. Such an explanation accounts for the very small percentage of parallel cleavages. If the spindle starts to form obliquely, the proto-plasmic movements are unequal about the aster and these centers tend to move into more radially symmetrical positions in the long axis. This accounts for the conspicuous absence of oblique cleavages after centrifuging.

Effect of centrifuging on the spindle

In the experiments relative to the mortality under various conditions, described above (table 1), two sets were centrifuged respectively forty-five and fifty minutes after fertilization at a time when the first cleavage spindle had formed in all the eggs. The percentage of mortality in these two sets was even less than in the control. Again a set of eggs was centrifuged forty minutes after fertilization and a single field was observed under the micro-scope until the 84- or 128-cell stage. Of ninety-five eggs in which cleavage could be seen all divided perpendicularly to the layers and eighty-eight developed to the late cleavage stages. The others divided irregularly and died; twelve eggs, which had divided perpendicularly to the stratification within ten minutes, were isolated. One died soon after isolation. The other eleven developed to the stage of swimming gastrulas. The development of the egg is apparently not interfered with by centrifuging in these stages.

A section of an egg centrifuged forty-five minutes after fertilization, killed immediately in picro-sulphuric and stained with Delafield's haematoxylin and orange G is shown in fig. 11). The spindle lies in the purple zone close under the cap and parallel to the stratification. Of forty-two spindles recorded in this position, six were equatorial plates, ten were in metaphase and twenty-six in anaphase. A great many more eggs were studied in an effort to find a spindle lying perpendicular to the layers. No evidence of such a condition was found, which bears out the evidence already recorded in the living eggs, where the cleavage is always perpendicular to the layers.

The spindle does not occupy its normal position in center of the egg but lies toward the top in the protoplasmic band just under the cap. The first question is in regard to orientation. The constant relation which Morgan described between the micropyle and micromeres showed beyond doubt that the unfertilized eggs do not orient themselves in the machine. It was possible, however, that the case might be different after the spindle had formed. As a means of testing the point, I centrifuged eggs in the late 2-cell stage, and found that the stratification bore no definite relation to the first cleavage plane; that is, the layers were in some eggs perpendicular to the cleavage plane, in others parallel or oblique. Some of the eggs were killed at once and found to contain spindles. The eggs, therefore, do not orient themselves in the spindle stages of the 2-cell egg and I see no reason to suppose that they do so immediately before first cleavage.

Since the eggs do not orient themselves in the machine and yet the spindles always maintain a definite relation to the centrifugal layers, it follows that in many eggs the spindle will not chance to be perpendicular to the line of force and in such cases something more than a pushing to one side of the karyokinetic figure occurs. The most striking evidence of this in the sea urchin occurs in the spindle stages of the 2-cell stage described above. When the stratification is perpendicular to the cleavage, the spindles are parallel to the layers and to the cleavage plane and consequently to each other. If the stratification is parallel to the cleavage plane

the spindles are likewise parallel to the layers and the first cleavage, but they are not always parallel to each other, which is an unusual condition.   However, the eggs in which the stratification is oblique to the cleavage are yet more unusual for the spindles are always parallel to the layers and not to the cleavage plane.   They may also be perpendicular to each other.   The results mean certainly that the spindle is not only shifted to one side but is in many cases swung through a considerable arc into its new position.

The second cleavage in centrifuged eggs where the layers are oblique to the first cleavage plane offered an opportunity of testing the influence of stratification upon the direction of cleavage. A number of eggs were observed.   In every case the second cleavage was perpendicular to the first cleavage.   There is only one meridian through which a plane can pass which will be perpendicular to the oblique layers as well as to the first cleavage.   In some cases the second cleavage cut through that meridian.   When this was not the case a shifting of the layers gradually took place, so that when the second cleavage cut through, the layers had become parallel to the first cleavage plane.   The second cleavage plane was then perpendicular to the layers as well as to the first cleavage.

To find that the cleavage spindles can be moved, although the moving be nothing more than a shifting of the whole figure to one side, bears on our conception of the structure of the karyokinetic figure.   That the substances of the egg are subject to a greater condition of tension or "rigidity" during karyokinesis than in the resting stages must be true for the materials of the egg are very much harder to separate at that time.   Nevertheless the spindles contained in eggs, killed immediately after centrifuging, are to all appearance uninjured, despite the fact that they have been at least pushed from their normal position in the center of the egg and often rotated into a position parallel to the layers. The eggs are spherical after centrifuging as they are normally and this probably accounts for the absence of any distortion in the general shape of the spindle such as has been described in cyclops.

The fibers of the spindle are as distinct as they are in the normal egg and there is no sign of tearing. The chromosomes are not displaced and the asters occupy their normal positions at the end of the spindle. The astral rays pass out into the surrounding protoplasm and there is no sign of modification of their perfectly radial arrangement. This is the strongest evidence against the theory that they are true fibers. A radial system of fibers could hardly be supposed to remain undistorted after being pushed through the egg.

The spindle was centrifuged in all stages, even as late as the time when the chromosomal vesicles are forming. The result was always the same. The nuclear elements lie under the oil cap. Eggs killed ten to fifteen minutes after centrifuging show that in that time there is no return of the spindle toward the center of the egg.

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EXPLANATION OF FIGURES

1 Cyclops fimbriatus—centrifuged egg, showing the spindle with one pole imbedded in the yolk hemisphere.

2 Cyclops fimbriatus—egg centrifuged longer than egg shown in fig. 1. Spindle parallel to the layers.

3 Cyclops fimbriatus—section of egg sac showing distorted spindle in *b* and *c* and the astral discs.

4 Cyclops fimbriatus—egg centrifuged in the anaphase of the two-cell stage, showing a longitudinal section of the spindle in one cell and a cross-section of one of the asters of the spindle in the other cell.

5 Cyclops fimbriatus—egg centrifuged just before the second cleavage. The layers and spindles are oblique to the first cleavage.
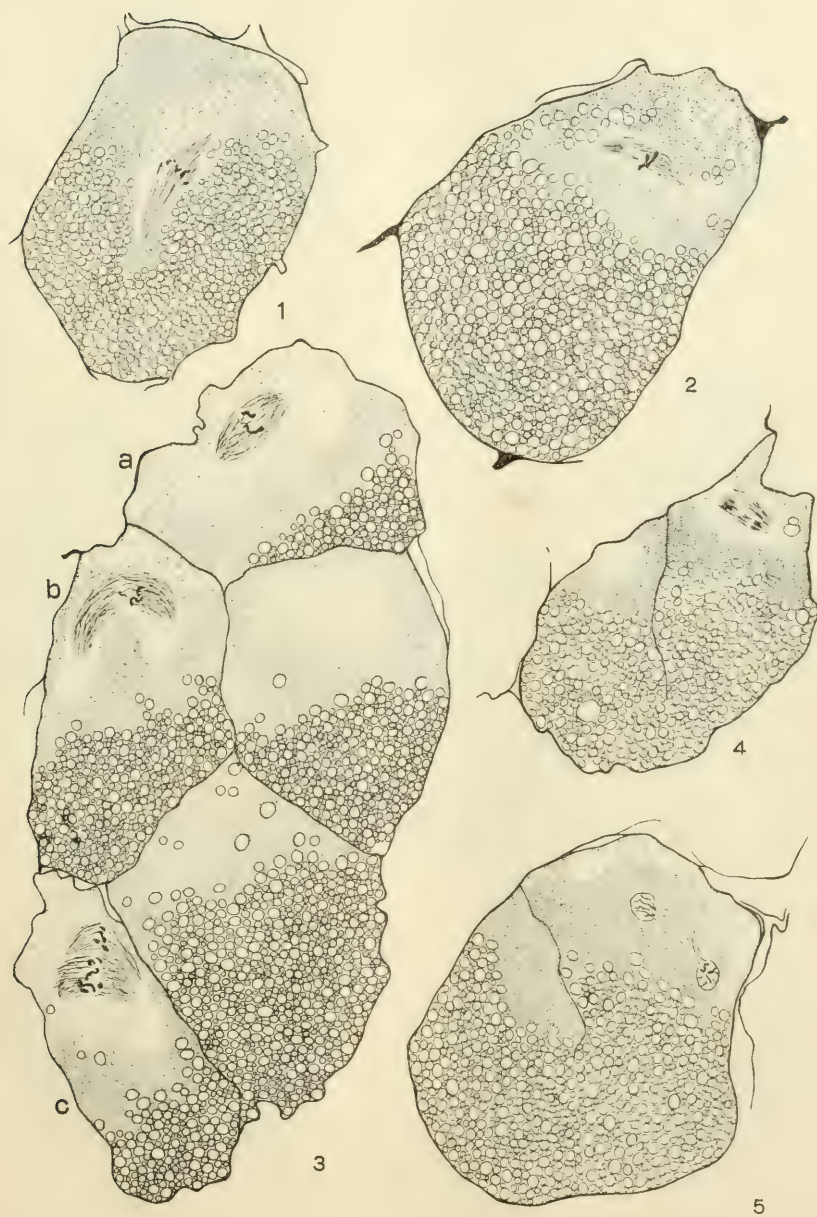
6   Cyclops fimbriatus—egg showing the second polar spindle lying near the periphery of the egg in a ball of protoplasm.

7   Cyclops fimbriatus—first cleavage spindle just forming, showing asters and astral rays.

8   Cyclops fimbriatus—egg sac after centrifuging, showing three layers, of yolk, protoplasm and oil.

9   Arbacia punctulata—egg in india ink solution.   The micropyle is not directly opposite the micromeres.

10   Cyclops fimbriatus—egg sac centrifuged after first cleavage, showing that the layers may bear any relation to the first cleavage.

11   Arbacia punctulata—section of an egg centrifuged just before the first cleavage, showing the spindle parallel to the stratification.

12   Arbacia punctulata—section of an egg centrifuged before fertilization. The spindle has formed in the short axis of the protoplasmic layers, perpendicular to the stratification.
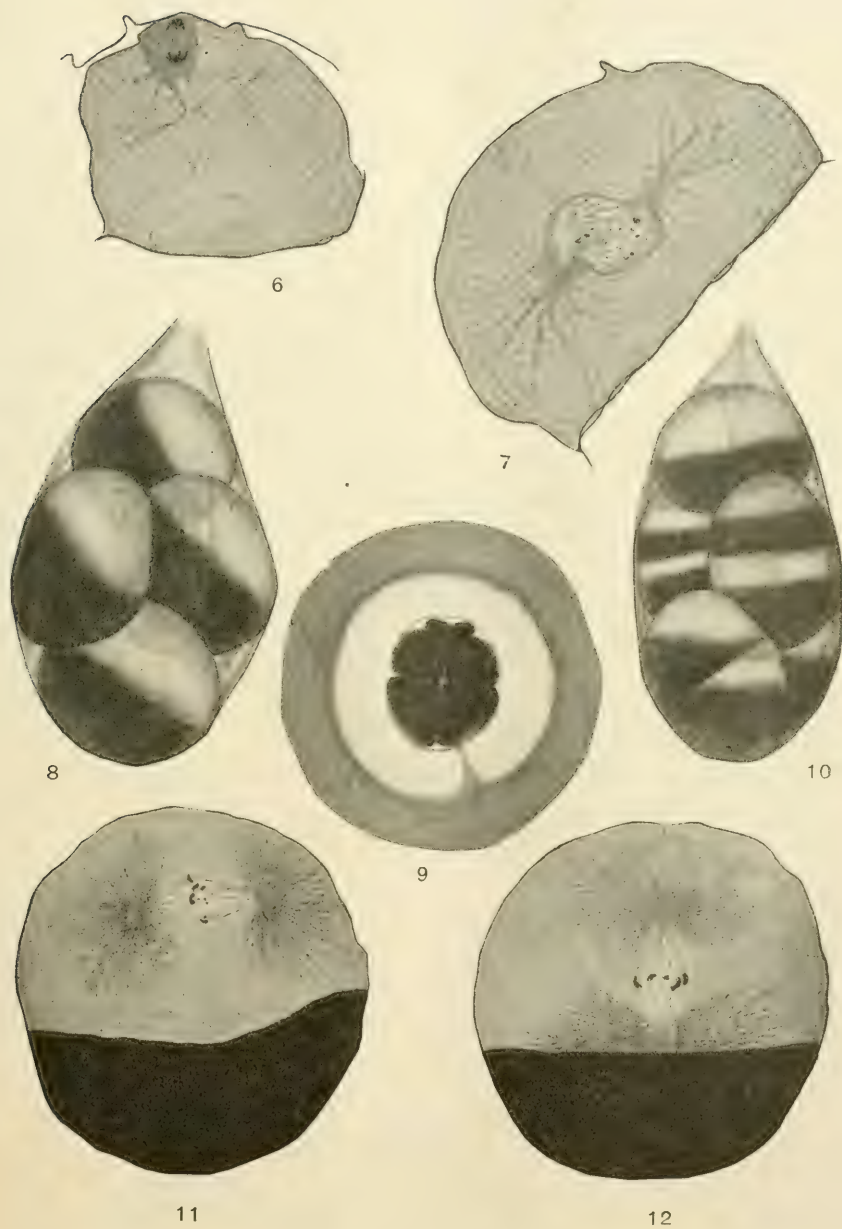
THE SENSE OF SMELL IN SELACHIANS

RALPH EDWARD SHELDON

I. INTRODUCTION

Fishermen and others interested in the question of bait or the feeding habits of fishes, have long held and expressed the opinion that many species obtain their food partly or entirely through the use of the sense of smell. Such have observed that, while the more agile among the teleostean fishes clearly avail themselves of their eyes in the pursuit of food, many species, both among the Teleosts and Selachians become acquainted with the proximity of food in a different manner. Some feed only in the dark, while others possess eyes, so small and imperfect as to be of little assistance. In still other cases the method of search indicates the use of another sense, as for instance the carp, ploughing through the mud and grass; the catfish, with its barbels trailing over the bottom, or the skates and dogfish which carefully search the sea floor with the ventral surface of their snouts in close proximity to it. Fishermen say that these species secure their food by the use of "smell," or possibly "taste," in the case of carp and catfish. Writers on the subject make similar statements (see Bateson, '90). When these are carefully analyzed, however, it will always be noted that the term "smell" means, in the minds of such observers, the recognition of food by means of a chemical sense. This so-called sense of smell may, then, consist of smell, taste, or a more general chemical sense.

Herrick ('02) shows that many species of Teleosts, such as the Cyprinoids and Siluroids, which are well supplied with taste

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buds on the outer surface of the body, particularly on the barbels and about the head (Herrick, '03), use these organs in the cognition of food substances in contact with them. The sense of taste must, therefore, be taken into consideration.

Sheldon ('09), finds that the reactions of the nasal apparatus to essential oils and to a number of other irritating substances, is due to stimulation of the mandibular nerve and not the olfactory, as was supposed by Nagel ('94). Sheldon also shows for Selachians, as Parker ('08), had earlier pointed out for Ameiurus, that the general cutaneous nerves react generally to many sapid substances. This may easily explain the refusal of fishes to eat food covered with essential oils (Aronsohn, '84). A general chemical sense is, therefore, to be accounted for.

Negative results after operations, such as the refusal of Scyllium to eat after destruction of the olfactory crura or lobes (Steiner, '88), are valueless, without the proper controls, as the shock of the operation may easily so disturb the fish as to prevent feeding. That this is likely to be the case is indicated by experiments carried out in connection with this investigation, where it was found that Selachians are extremely sensitive, so far as their feeding habits are concerned, to any interference with their normal life or environment. Physiological evidence regarding the function of the olfactory apparatus of aquatic vertebrates has then, until very recent date, been negligible. It is also clear that the ability of such an animal to recognize food at a distance does not necessarily mean the use, therefor, of the sense of smell.

Anatomists and neurologists, struck by the constancy and size of the nasal apparatus of fishes, as well as by the complexity and long phylogenetic history of the nervous mechanism by which it is brought into relation with the different parts of the brain, have long believed that it must be an apparatus of paramount importance in the life of the individual, particularly with respect to the recognition of food substances (see Johnston, '06).

In the last number of volume 8 of the Journal of Experimental Zoölogy, appears an article by Parker, recording for the first time, in the Siluroid, Ameiurus nebulosus, reactions unquestionably due to stimulation of the olfactory apparatus. He finds that

when two packets of similar appearance, one containing minced earthworm, the other empty, are placed in a jar containing catfish, these invariably seek and tug at the one with food. Fishes with the olfactory tracts cut never respond in this manner. Observations by Dr. Parker,[2] on the sense of smell in Fundulus, give similar results and show also that the failure of the fishes to respond after section of the olfactory tracts is not due to the shock of the operation. In Fundulus, however, the eyes are also used in the recognition of food substances.

II. THE NASAL APPARATUS OF THE DOGFISH

This consists of a pair of large capsules, partially divided into two parts by means of a superficial and an enclosed flap of skin rostrad, and a fleshy ridge caudad. There are thus two incompletely separated external apertures, a rostro-lateral and a caudo-median, the latter the closer to the mouth (see Sheldon, '09, fig. 3). These capsules contain a double row of lamellae extending laterad from a median ridge, much as do the barbs from the rachis of a feather. This median ridge extends from the more lateral to the more medial opening. The lamellae are innervated by an enormous number of short olfactory nerve fibers which terminate in the large olfactory bulbs, closely apposed to the capsules. The nervus terminalis of Locy also sends a few fibers into the lamellae, while the capsules, in general, are innervated for tactile and general chemical sensation by the nervus maxillaris trigemini.

During the ordinary movements of respiration, as water is taken into the mouth, a current is, by suction, drawn through the nostrils, entering at the more rostral and leaving at the more caudal aperture to be drawn farther, to some extent, into the mouth. This may be easily demonstrated by fastening a dogfish on its dorsum and expelling, from a pipette, a colored solution rostral to the nostrils. The current then follows the median ridge, a part being diverted laterad between the lamellae. The shape and position of the fleshy ridge and flaps of skin are such,

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[2] See this number of the Journal, page 1.

also, that a fish in forward locomotion forces water through the nostrils. A dogfish is, therefore, whether in motion or at rest, constantly receiving through its nasal capsules, a current of water.

III. THE REACTIONS OF THE DOGFISH TO OLFACTORY STIMULI

The experiments here recorded were performed on the smooth dogfish, Mustelus canis (Mitchell). This was selected owing to its great abundance in Buzzard's Bay near Woods Hole, and also because previous experimentation (Sheldon, '09) rendered many of its habits and reactions familiar.

Feeding habits

Bateson, Nagel, and others believed that Selachians recognize their food through the sense of smell; their evidence, as has been noted is valueless, however, in this connection. Mr. Vinal Edwards, Collector at the Woods Hole Station of the Bureau of Fisheries, states that it is the custom, in fishing after dogfish, to throw out in the tide lines baited with menhaden or alewives. For a time no dogfish will be seen, then they will appear in numbers, swimming around the bait in gradually diminishing circles until finally it is seized. Field ('07), finds that the dogfish carefully search the bottom for crabs. Finding one, they turn on their sides to seize it, then dart off swiftly, shaking the crab as a terrier would a rat. After swallowing the food, Field states that the dogfish keeps up its active swimming, often returning to the place where the crab was found.

Some experiments on the relation of the olfactory apparatus of the dogfish to its feeding habits were undertaken in the summer of 1908 but failed owing to the fact that the fishes refused to eat in captivity. These experiments and those of Field show that dogfish will not eat if kept in large tanks or even in the large cod cars of the Station, even though they are kept in captivity to the point of emaciation. In order, therefore, to give them a habitat comparable to the normal, a portion of the large observation pool of the Station was fenced off with meshed wire. This gave a pool

24 feet long, 8 feet wide at one end and 10 feet wide at the other, with normal sea bottom, an irregular stone wall on three sides, and a depth of water of from two to eight feet, depending on the portion of the pool considered and the height of the tide. Tufts of eel grass, together with many other varieties of sessile marine life, grew on the bottom and sides. The pool, therefore, fulfills, to a reasonable degree, the conditions of normal life, so far as the dogfish are concerned.

The individuals used were those caught in the traps from day to day, and placed in the pool for a period of ten days in order to bring about a state of hunger.

Experiments with normal fishes

Spider or blue crabs were first offered the dogfish, but were always left untouched. The rock crab, Cancer irroratus, was next tried with success, and used for all the experimental work. All experiments were conducted at low tide when it was easy to observe the actions of the individual fishes. At first living crabs were used. These are found by the dogfish in from ten to fifteen minutes. Next, crabs were killed and a hole broken in the cara-place, exposing the flesh. Such are found in from two to five minutes. These results suggest, at the start, that food is recog-nized through the diffusion of animal juices into the water. Crabs killed, with the flesh thus exposed, were used for all further work.

In a total of about forty experiments the method of feeding was the same in all cases. The dogfish spend most of the time swim-ming lazily around the pool, usually close to the sides. Now and then the direction is reversed, but at no time was there observed any search over the bottom or the rocks forming the sides of the pool. When a crab is placed in the pool a few minutes are re-quired, as noted, before any evidence of stimulation is to be seen. Then one of the dogfish, which happens to be swimming within three or four feet of the crab, seems suddenly startled. It turns very suddenly and swimming with quick, nervous motions, instead of the calm, lazy movement of the unstimulated fish, be-gins a systematic search over the bottom, investigating particu-

larly grassy or uneven spots. The head is moved rapidly from side to side as the fish swims slowly, coursing in gradually diminishing circles, two or three inches from the bottom. When within two or three inches of the crab the dogfish seizes it suddenly, making off in a swift rush. As remarked by Field, the crab is shaken violently from side to side for a moment, as the shell is crunched and broken by the powerful jaws of the fish, after which it is quickly swallowed. Occasionally, however, the crab is dropped during the process. When this occurs a search, similar to the first, follows until it is found again.

At no time did the dogfishes appear to make any use of the sense of sight in feeding. A crab hidden in eel grass is found as quickly as one lying on the open bottom; moreover, one is found equally quickly whether lying in its venter, exposing the dark carapace, or on its dorsum, with the light colored venter showing conspicuously. A dogfish, dropping a crab, is apparently absolutely unable to find it again excepting by means of the same sense which enabled recognition of food in the first place. It was observed, however, that a dogfish with food is usually followed by others in the vicinity, which endeavour to secure possession of it. Moreover, the fish will frequent for some time thereafter the region of the pool in which food was found. This is probably due to olfactory stimuli, although sight may be brought into play to a slight extent. It was often noted that a dogfish would circle around the spot where a crab had lain, often biting into the bottom at the exact spot; probably some body juices had escaped into the ground. Now and then a crab was placed on the bottom near the screen separating the experimental from the larger pool. If no fishes, capable of finding food, were present in the former, it would often happen in a few minutes that eight or ten dogfish from the large pool would be swimming rapidly back and forth along the screen, endeavoring to find their way through.

Some experiments were tried with a hook and line baited with the flesh of a rock crab tied in cheese cloth. The dogfish here followed the same procedure as when the crab lay on the bottom. On noting the proximity of food the fish begin to swim, as before, in circles but, for a time, persistently search the bottom beneath

the baited hook. At length they gradually rise, turning some-
what sidewise, as stated by Field, to seize the bait. When the
food is lying on the bottom this sidewise turning was never ob-
served.

Observation of the feeding habits of the dogfish would indicate,
then, that it recognizes its food through some chemical sense.
To test this the following experiments were performed.

Some fresh eel grass was secured and two packets, closely re-
sembling each other were made, one containing a small stone
while the other enclosed a crab. Both were so tied that when
placed in the water a foot apart, a portion of the grass rose toward
the surface giving an appearance similar to the grass of the pool.
In three sets of experiments the presence of food was detected
in an average time of three minutes, the packet found by the usual
procedure, torn apart, and the crab eaten. At no time did the
packet containing the stone receive the slightest attention.

Next two packets of white cheese cloth of a similar size and
appearance, were made up. One of these contained a stone, the
other a crab. This experiment was repeated four times on differ-
ent days. The packet containing the crab was found in each
case in from three to five minutes while the one with the stone was
never molested. The two packets were placed from ten inches
to three feet apart. Once or twice a dogfish which had eaten a
crab would return and, circling about the spot where the crab
had been found, would approach the packet containing the stone
in an inquiring sort of way but at no time touched it. The packet
containing the crab was always shaken and bitten until the food
could be removed and eaten.

A crab was killed and a piece of white cheese cloth saturated in
its juices. This was then attached to a small stone. In two
experiments the presence of a food substance was noted in two
minutes. The stimulus was located by the usual circling method
and the stone, with its saturated cloth, seized again and again
and shaken violently . After a half hour the fish took no further
notice of it, probably due to a complete diffusion of the juices into
the water.

In all the above experiments a number of different sets of dogfish were used. There were usually from six to eight fish in the pool at a time. Usually only one or two experiments a day were performed in order that there should be no interference between them.

These observations show, beyond doubt, that the dogfish obtains its food through the use of a chemical sense. Experiments were now undertaken to find out what part the olfactory apparatus plays in these reactions.

Experiments on fish with occluded nasal apertures

Four dogfish which had eaten readily when in the normal condition, were removed from the pool and their nostrils stuffed with cotton wool; in two of the cases the cotton was covered with vaseline. When returned to the pool such fishes rush about violently for a few minutes, as do all dogfish which have been out of water, They soon, however, quiet down and swim about the pool as do the normal fish. Twenty-four hours later three crabs were placed, an hour apart, in the pool, which now contained, in addition, four normal fishes. All were found, in the usual manner and length of time, by the fishes without cotton in the nostrils. At no time did any of the individuals with the nostrils filled show the slightest interest in the crabs, although such often swam within a few inches of the food. Moreover, these fish made no attempt to follow those which had secured one of the crabs, although the food was occasionally dropped. It was often observed that two dogfish, one normal and the other with the nostrils filled, would be swimming along the wall side by side when they approached the vicinity of the crab; the normal fish would then make the usual sudden turn to search for food while the individual with the cotton continued on its way with no change in the lazy swimming movement. As it was noted that vaseline or a close packing of the nostrils with cotton caused suppuration in time, this experiment was repeated three times with cotton loosely packed. Results, similar to the above, were secured in all cases. Two tests

were made, also, in which there were no normal fishes in the pool. In these cases the crabs were left untouched for twenty-four hours, although the dogfish on the opposite side of the screen became much excited.

Three of the dogfish, all of which had eaten readily before the use of the cotton, but which had refused to do so thereafter, although tested for three successive days, were removed from the pool and the cotton withdrawn from the nostrils. These were returned to the pool which now contained no normal fishes. The following day one of these ate readily in the usual manner, although there seemed to be slightly more difficulty than usual in finding the crabs, both in the first place and after one had been dropped. A day or two later all three ate as usual. This experiment was repeated twice with the same results.

These experiments indicate that the dogfish normally recognizes the proximity and location of food through the use of the olfactory apparatus. It may be argued, of course, that the mere presence of the cotton in the nostrils renders the fish so uncomfortable that it refuses to eat, even though it acts otherwise in a perfectly normal manner. To obviate this objection four dogfish were removed and one nostril only stuffed rather tightly with cotton. These four were now placed by themselves in the pool. One of them, within an hour thereafter, caught a crab, after the usual preliminary procedure, but lost it and seemed to take no further interest in the matter. The following day all four ate as usual. It was noticed in this experiment also, that the dogfish had rather more difficulty than was normally the case in finding the food, but that this wore off in a couple of days. These four fish were removed after four days and seven others, treated in the same way, were substituted. The results were the same. These tests show that the presence of the cotton is not sufficiently irritating to interfere seriously with the normal feeding habits of the dogfish. As remarked, earlier individuals with the nostrils plugged act, except in so far as the feeding habits are concerned, just as do the normal fishes; as both kinds swim about the pool, such could not be identified by an uninformed observer.

IV. CONCLUSIONS

The experiments and observations here recorded show that the passage of a current of water through the nasal capsules of the dogfish is necessary for the recognition of food substances and the identification of their location. The nerve termini stimulated by the food juices dissolved in this current are, with little question, olfactory. Experiments performed on dogfish with the olfactory crura cut, but with the mandibularis nerve intact, show that such individuals do not react to food juices, but to tactile and general chemical stimuli only. The function of the nervus terminalis is unknown, but the number of its fibers is very small and anatomically it gives no indication that it is of special importance. It is, moreover, practically negligible in the case of the teleostean fishes which react to the stimuli of food substances in a closely similar manner (Parker, '10, '11).

The statement is sometimes met with that the the function of the olfactory apparatus of aquatic vertebrates can not be similar to that of the terrestrial forms as in the one case the stimulating substance is in solution in the water, while in the other it is floating in the air. As all substances which the air breathing vertebrates recognize by means of their olfactory apparatus must first be dissolved in the moisture of the nasal mucous membrane, there is no distinction between the two cases.

Olfactory sensation differs from general chemical and from taste in that the stimulating substances are in more dilute form; it is, therefore, chiefly for the cognition of distant substances, as Sherrington ('06), Herrick, ('08), and Parker, ('10) have pointed out. It may then be stated that, on the evidence here submitted, the olfactory apparatus of the dogfish reacts to substances in so dilute a solution in the water that the taste buds and other organs of chemical sense are not affected. The selachians, then possess a sense of smell comparable to that of terrestrial vertebrates.

V. SUMMARY

1.   A current of water, caused by respiratory movements, and augmented by the forward motion of the fish in swimming, courses through the nasal capsules of the dogfish.

2.   By this means substances in solution in the water come in contact with the olfactory mucous membrane.

3.   Dogfish recognize and determine the location of food substances through a chemical sense.

4.   This power is lost when the olfactory capsules are filled with loose cotton.   It is regained when the cotton is removed.

5.   The plugging of one nostril only does not seriously affect this power.

6.   The dogfish obtains its food almost, if not entirely, through the sense of smell.

7.   The Selachians possess a true sense of smell, comparable to that of the terrestrial vertebrates.

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THE GROWTH OF TISSUES OF THE CHICK EMBRYO OUTSIDE THE ANIMAL BODY, WITH SPECIAL REFERENCE TO THE NERVOUS SYSTEM

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The relation of fibrin to the healing of wounds in the animal body, to the formation of adhesions in serous cavities has long been known to pathologists. Clots of blood and serous fluid occurring in various forms of injury, act, according to our present knowledge of the subject, as a supporting framework on which connective tissue, blood vessels and the epithelium grow to bring about the healing of a wound.

The use of fibrin clots outside the body as a culture medium for growing embyronic tissue, we owe to Harrison ('07–'10). This author has conclusively proved, by means of a long series of experiments, that embyronic tissue of the frog will undergo development of a normal type for a considerable period of time, when transplanted into a coagulable lymph. The isolated central nervous system of a frog-embryo, removed to a cover slip at a period of development just prior to the appearance of peripheral nerves and covered with fluid lymph from the lymph sac of an adult frog, is firmly held by the coagulating lymph and passes through a period of considerable development, growing long nerve fibres. These fibres, as this author has described, grow in the meshes of the fibrin by an independent activity of their own

protoplasm and are in no way actively influenced by the changes taking place in the clot.

The opponents of the doctrine of the independent outgrowth of nerve fibres from a central neurone, as advanced by Kupffer and His and so strongly supported by Cajal, Harrison and others, have questioned, on grounds which need not be detailed here, whether the fibres growing in lymph observed by Harrison were really nerve fibres and whether they could be shown to be made up of neuro-fibrillae, which take the specific stains that form the basis for neurological study at the present time.

Harrison has answered these objections in his latest paper and by a full analysis of his experiments, has shown with all reasonable conclusiveness that the structures in question are nerve fibres, but he has nevertheless pointed out the desirability of showing the identity directly, by means of specific staining reactions. While the present investigation was not directed especially to this end, it has nevertheless been found possible to obtain satisfactory permanent preparations of the artificially grown nerve fibres and by means of Held's molybdic acid haematoxylin to demonstrate the neurofibrillae with remarkable clearness.

The main purpose of the work here presented was to study the movement, growth and differentiation of the embryonic tissues of a warm-blooded animal and to analyze the conditions of environment which would stimulate or inhibit their independent development. In proceeding with such an analysis, it has been found necessary, as Harrison ('10) had already pointed out, so to improve the techinque that culture media of constant composition could be obtained and then modified at will. This has been successful beyond expectation, and results which, it is believed, are of considerable interest have been obtained, though time has not yet permitted the carrying out of a full set of analytical experiments.

The main results of the work have been announced briefly in two preliminary communications read before the Société de Biologie (Burrows, '10), and since then the author, in collaboration with Dr. Carrel ('10), has described the results of some experiments with adult tissues which were made after the present investi-

gation was completed. In the present communication the technique of preparing the tissues and the culture media and the observations upon the growth of the isolated embryonic tissues of the chick embryo, will be taken up, leaving for future work the full analysis of the conditions which affect this growth.

MATERIALS

Chick embyros are of special advantage, not only on account of the ease of obtaining them throughout the year, but also on account of the importance of having a warm-blooded animal for this study.

Secondly, the tissues of these embryos receive their nutrition at an early age from an extracellular yolk through a well established vascular system. The removal of the tissue from the embryo interrupts its vascular connections and removes all the nourishing agents of the yolk. The development, therefore, of such a technique offers not only the opportunity for the study of tissue growth, as originated by Harrison, but also gives an opportunity for the study of cell nutrition.

For the work at hand embryos of sixty hours of incubation were found most suitable. At this stage, according to Held ('09), some peripheral nerves have formed. However, there are many neuroblasts which have not begun their differentiation.

At a culture medium blood plasma prepared from the blood of adult chickens was substituted for the lymph used by Harrison, and in this way the chief difficulties encountered by Harrison were overcome. This medium was easily obtained, and the clots were firm and uniform, giving a greater range of control. Such preparations could be readily preserved and stained by any of the usual histological methods.

METHODS

Dissection of the tissues

The methods of dissection of the nervous system, muscle plates and other tissues were similar to those used by Harrison in the frog embryos. The preservation of the specimens from bacterial infection has been a comparatively simple matter. The observance of simple surgical technique and the use of freshly sterilized glass-ware and instruments for each operation are sufficient. To prevent long periods of chilling, the solutions and dissecting watch glasses were kept at 39° C. The dissections were made under a binocular microscope, which was completely covered by a warm oven, heated to 39° C.

The course of an operation for the preparation of specimens from an embryo may be summed up as follows: The egg is taken from the incubator, and the embryo in its membranes quickly removed with sterile instruments to warm Ringer's solution under the binocular microscope. The membranes are removed. The heart, now quite free from the surrounding tissues, is next removed with scissors by cutting through the base of the vitelline vein and the truncus arteriosus. The dissection of the nervous system is now begun. The specimen is balanced with forceps; two lateral incisions of the skin are made with scissors just ventral to the nervous system; the optic cups are excised close to their connections with the neural tube; the skin and the mesenchyme are carefully removed with needles; the cord is now cut in the upper cervical region and this piece of neural tube, comprising brain and upper cervical cord, is floated off free into the solution. The tube shows a round normal shape, open at the points of attachment of the optic stalks, at the end of the cut cord and at the roof of the fourth ventricle, which has been removed with the skin during the dissection. Brains were transferred to plasma in this condition in all the early experiments. In later preparations a hemi-section of the nervous system was made with sharp scissors along the median sagittal plane. The reason for this procedure will be discussed later.

The muscle plates were dissected out in a somewhat similar manner. The skin and the surrounding tissue were removed and the muscle plates were cut free from the neural tube with sharp scissors.

The piece of tissue to be transplanted was taken from the Ringer's solution with a small sterile pipette and dropped with the solution on the surface of the cover glass. Excess of Ringer's solution was removed with the pipette and a drop of the plasma run over the specimen. The cover glass was then either immediately inverted over the hollow slide or the slide placed over the surface of the cover glass, depending on the position desired for the specimen in the drop; the specimen settled always to the lower surface of the drop. The cover glass was then sealed to the slide with paraffin. Clotting took place at once before the slide could be transferred to the incubator. The time occupied for the entire dissection and mounting was five minutes.

The preparation of blood plasma

Oxalated plasma. In the earlier work on chicks, some difficulty was encountered in the preparation of a pure plasma which could be preserved for sufficient time to allow for the dissection of the specimens. In searching for an agent which prevents coagulation, sodium oxalate was selected as the most suitable.

The blood was removed with a pipette from the heart of a chicken to a test tube containing 1 per cent sodium oxalate solution. Sufficient blood was added to make the concentration of the sodium oxalate 0.1 per cent. The tubes were then centrifugalized and the clear plasma removed with a pipette and placed on ice until ready for use.

For using this plasma, quantitive estimates were necessary for the precipitation of the sodium oxalate with calcium chloride and for the correction of the excess of the sodium chloride resulting from this chemical change. The precipitation was made, as the drops were used, in a pipette graduated to one part in ten or one part in five, depending on the dilution of the plasma desired. The quantity of calcium chloride necessary to precipitate the sodium

oxalate was made up in Ringer's solution which contained the
desired lower concentration of sodium chloride. One part of this
solution precipitated the sodium oxalate in nine parts of the blood
plasma.

In the first series of preparations of thirty-five specimens, eleven
showed active growth of mesenchyme cells for the first forty-eight
hours. Magnesium was added in various concentration in the
next series, one part in fifteen thousand proving most satisfactory.
In all specimens of this series, forty-three in number, marked cell-
ular activity of mesenchyme elements was noted throughout
the first forty-eight hours (fig. 2). After this time, all showed
degeneration and death. The addition of magnesium in this series
gave marked increase in the number of specimens developing in
oxalated plasma, but no increase in the duration of growth. Sub-
sequent control series gave the same results. When aqueous
extracts of the area vasculosa, together with ether-soluble bodies
of the yolk, were added to this plasma in small quantities, an
increase in duration of growth was the result. The specimens of
this type showed a period of growth extending over seventy-two
hours, but no nerve fibres developed in these preparations.

Pure plasma. The use of a pure plasma has given far better
results. In this medium, the growth was prolonged for a number
of days with marked activity of both the mesenchymatous and
epithelial tissues. In the following descriptions, only specimens
grown in this medium have been used.

The method of preparation of pure plasma has been in part
similar to that used by Delezenne ('97). The blood was obtained
from young healthy chickens by ordinary operative procedure.
Ether was used as an anæsthetic. The carotid artery was exposed
and a canula, previously sterilized in pure olive oil, was inserted,
necessary precautions being taken against contamination of the
blood by the tissues (Delezenne). The blood was collected in
thick-walled paraffin-coated tubes and quickly cooled by placing
the tubes in an ice-salt bath. The tubes were centrifugalized at
once by placing them in a large centrifuge tube filled with ice and
salt. The supernatant plasma was then removed with a paraffin-
coated pipette to another tube and placed in a refrigerator until
ready for use. Plasma may be obtained by this method in a very

stable condition as far as coagulation is concerned. On this account, plasma which would coagulate at summer temperature in twenty minutes or less has been most satisfactory for the present work. The drops on the side coagulate very rapidly at 39° C. and hold the tissue firmly. When it is lacking in this ability to clot spontaneously at high temperature, it often remains fluid for several hours and the clots about the tissue lack uniformity and firmness with a corresponding disturbance in uniformity of growth. No plasma over four days old was used for any experiment.

GENERAL DESCRIPTION OF THE EXPERIMENTS

The different tissues of the chick embryo, when isolated and transplanted to a coagulable plasma, show marked activity of proliferation and growth for a considerable period of time. In the present set of experiments, the tissue of the central nervous system was especially studied, other tissues being used as controls for further proof in the identification of cells and nerve fibres.

The tissues most active, as shown by table 1, have been the nervous and mesenchymatous tissues. Muscle cells show little evidence of growth.

TABLE 1

TISSUES	NUMBER OF PREPARATIONS	NUMBER OF PREPARATIONS SHOWING		
		Muscle cell	Nerve fibres	Mesenchyme cells
Whole neural tube	64	0	3	64
Hemisected neural tube.......	47	0	23	47
Teased pieces of neural tube..	27	0	21	27
Heart.......................	40	3	0	36
Myotomes....................	12	4	0	12

The first appearance of cellular activity in any of the above tissues appeared between the second and the twelfth hours and generally not later than the end of the first day. This early cellular growth was always identified as mesenchymatous in origin. The growth of nerve and muscle cells generally begins to show between the second and sixth day after transplantation. The cellular activity continued from six to eight days in most of the preparations. Some few remained active as long as twelve days,

when degeneration was noticed. Death may be very sudden, involving all the cells in a short period of time. In carefully handled specimens, however, the first signs of degeneration were near the tissue originally transplanted. The more distant cells died at a considerably later period.

The rhythm of the heart continues normal in the plasma unless it is disturbed by adhesions to the clot. As a general rule, the cut ends of this organ immediately become attached to the fibrin, first by adhesion, later by the extension outward of a large number of spindle-shaped mensenchyme cells. The rest of the organ, unless injured, gradually frees itself from its loose fibrin attachments through its constant activity and beats in an open serum cavity. These hearts beat for the first three days with a normal rhythm. After the third, the rhythm and the force of the heart are periodically altered. There is a gradual slowing of activity accompanied by alternate periods of acceleration and inactivity. The activity ceases completely between the third and the eighth day.[1]

The neural tube at the time of transplantation, as seen in preparations stained in toto and in serial sections, lies firmly held in a fine granular fibrin net. The fibrin lies for the most part without the tube. The central canal is filled with serum. The nerve cells are in their normal relations and react normally to stains. About the outside and closely adherent to the neural tube is a thin layer of mesenchyme cells not removed at operation. This layer of mesenchyme is very thin, generally one or two cells thick, and rarely continuous over the entire tube.

Examinations of a whole transplanted neural tube shows in gross after the third or fourth day a wide layer of cells growing in plasma about a well formed tube. In serial sections, the nerve cells are seen to be undergoing degeneration. The central canal is filled with dead cells and débris. The outer layer of mesenchyme cells, on the other hand, has developed into a layer many cells

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[1] Hooker has shown that the heart functions and develops normally in frog embryos after the nervous system had been entirely removed at a period before any nerve fibres had developed. If nourishment were renewed sufficiently often to these isolated hearts, function and development would undoubtedly proceed for a long period of time.

in thickness and the outgrowth of cells into the plasma can be readily traced to this layer. These cells are similar in every way to interstitial cells growing from the heart and the myotomes.

In 64 transplantations of whole neural tubes, only three preparations showed any activity of the nerve cells. The degeneration of the tube as studied in serial sections was commonly greatest in the cells most distant from the surrounding medium. Poor nutrition was the probable cause of this early death. Injury of the heart has most often been associated with the outgrowth of muscle cells. Injury to the nervous system would possibly disturb an equilibrium and stimulate activity. In the next series of preparations hemisections of the tube were transplanted in the hope of bringing plasma into close contact with both sides of the wall as well as of disturbing the continuity of its cells. Of the thirty preparations, 50 per cent formed long nerve fibres. In these preparations, the form of the neural tube is lost after the third day by an apparent separation of the cells. The mesenchyme layer tends rather to move outward into the surrounding plasma that to proliferate about the tube.

The exuberant growth of the mesenchyme cells in all these preparations covered the field of the growing nerves and hindered decidedly in determining whether such cells were necessary for nerve production. In the hope of obtaining pieces of the neural tube uncontaminated by these cells, the later experiments were made by teasing the tube into small pieces which were scattered throughout the drop. Many of such pieces, free from mesenchyme, sent out long nerve fibres into the clear plasma clot, fig. 8. The activity of the tissue is also increased by division into small pieces, when teasing is carefully done. The number of individual nerve cells sending out processes stands in inverse ratio to the size of the piece. Almost every cell in two pieces of ten and twelve cells respectively sent out nerve processes. The study of growth of the mesenchyme cells was also facilitated. One group of four cells had grown to a mass of fifty cells after six days of cultivation.

During the development of this technique for the study of nerve fibres in culture, various forms of growing mesenchyme cells have constantly been seen and some of the observations on the growth of both of these tissues will be discussed below.

NERVE FIBRES

Growth of the nerve cells is evidenced by filaments of various
sizes, which appear along the border of a piece of neural tube and
which grow out along a wavy course in the transparent clot. These
filaments vary in size from very fine threads to coarse cord-like
strands. The slender filaments are composed of a hyaline homo-
geneous protoplasm, while in the coarser bundles the homogene-
ous character is altered by the appearance of delicate, longitudi-
nal striations. The latter bundles break up into many fine fila-
mentous branches, either at their ends or along their periphery.
At the end of each of these growing filaments and branches is
the characteristic thickened amoeboid swelling as described by
Harrison. This is an oval or round swelling of the filament from
which protrude many actively moving delicate pseudopodia. The
growth of a fibre consists in the great prolongation and enlarge-
ment of one of these pseudopodia with a gradual moving out-
ward of the end knob along the pseudopod. The growth may be
so rapid that the end knob may entirely disappear, to reappear
farther out along the new grown part.

The growth of nerve fibers in any culture is always limited to a
short period, from forty-eight to seventy-two hours. During this
time they may grow very rapidly, a micron to a micron and a half
in a minute, and reach a length of from one to two millimeters.
Other nerves remaining active for a long period may never reach
any considerable length. The activity of such fibres is noted at
the amoeboid end and consists in a constant retraction and new
formation of pseudopodia. All observations on the movement of
the growing fibre suggest an active force within it causing its
extension into the medium.

True degeneration of the nerves has occurred in only two
per cent of the cases. This appeared in the form of nodosities
with some fragmentation after four days of growth. The
more common end of the life history of a nerve fibre is re-
traction. This phenomenon is noted a few hours after a complete
cessation of movement. It appears first as a pronounced length-
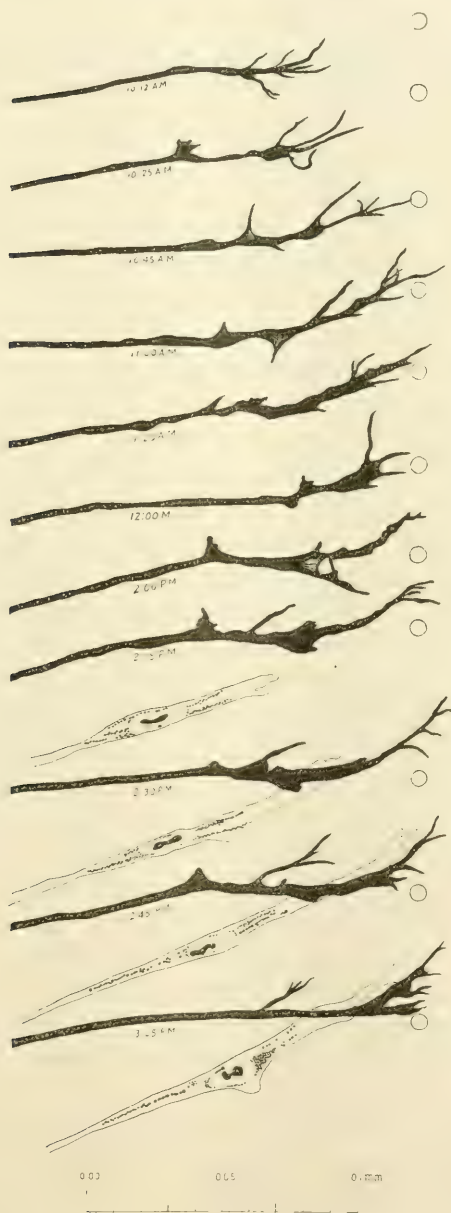ening of the thickened end accompanied by marked shortening

Fig. 1 Camera lucida drawings of the growing ends of a nerve fibre showing its relation to a constant point on the cover-glass. An amoeboid cell was included in the drawing after 2:00 P. M. Three day culture.

of the fibre. The shortening is generally rapid, greatly exceeding in rapidity the greatest rate of extension. The changes in the nerves are mainly at the end. Here there is periodic thickening, followed by a slow reduction in size until the entire nerve has retracted into the tissue in a manner similar to the retraction of the psuedo-podium of an amoeba. These phenomena of extension and re-traction may go on alternately in the same fibre. There is fre-quently extension of the fibre for a considerable period of time, followed by a period of quiescence and retraction. The retraction is checked after a time and growth again proceeds in a different direction for a while when the process is again repeated. This phenemenon is shown to some extent in fig. 1.

That these fibres are identical with the nerve fibres of the em bryo seemed without question from their general morphology and their origin from nerve tissues only. To complete, however, the general histological methods of identification, the reaction of the fibres to various stains, haematoxylin and Congo red, Cajal re-duced silver method and Held's molybdic haematoxylin, was de-termined. With both the Cajal and the Held methods the nerve fibres take the characteristics color. The Held stain, however, has been used for the preparations figured and described below on account of the greater contrast shown in the experiments stained by this method. The fibrin background has stained deeply with the reduced silver method and obscured consider-ably the slightly deeper staining nerve fibres.

The individual neuro-fibrillae are shown clearly in the stained preparations. The larger bundles appear as twisted rope-like strands or flat layers of delicate fibrillae (fig. 4). The very fine nerves stain more homogeneously, and often only a single fibril can be distinguished throughout their entire course. The end bulbs appear as faintly stained enlargements at the end of the axis cylinders (fig. 5).

The end bulbs and their pseudopodia stain irregularly, show-ing one or more dark staining bands which pass from the nerve fibre to the different pseudopodia (fig. 9). This dark-staining material may often be broken and appear as dark-staining globules, lying in the main mass of the bulb (fig. 5). In some cases the fibres

may apparently end without this enlargement. In many such cases an enlargement is seen far back along the fibre (fig. 10). This is apparently similar to the true end bulb.

A discussion of the literature on nerve development and a further comparison of these nerves with the nerves of the normal chick embryo will not be taken up, but as far as I have been able to determine, they conform in every detail to the nerves of the embryo, as a comparison with Held's ('09) excellent figures show. By a study of the stained serial sections of the culture, these fibres can be definitely traced to the neuroblasts as their cells of origin.

The segmental arrangement characteristic of the embryonic body has never been noted in nerves growing in culture from a carefully isolated neural tube. Nerves appear very irregularly along the pieces of transplanted tube and pass off irregularly into the surrounding fibrin network. The character of the movement and the course followed by a growing nerve suggests dependence on the chemical condition of the media. The physical relations, as shown in the architecture of the fibrin, apparently have little influence aside from slight changes in the form of the larger bundles. Nerves growing in a loose mesh or on the surface of the clot spread out in a flat layer of fibrillae (fig. 7) which separate very quickly into a large number of branches; these branches anastomose frequently and form a network. In the dense fibrin network they have a more compact rounded form. The possible existence of attractive forces between other tissues and growing nerves has been studied by transplantation of heart and myotome in close proximity to a neural tube, but in only one of forty preparations did the nerves enter the neighboring piece of tissue. Here the fibre apparently was directed along a dense band of fibrin which connected the heart with the point of exit of the nerve from the neural tube. The nerve fibres in no way influence the growth or the arrangement of the mesenchyme cells with which they are associated. The fibres assume the same course about these cells as they do in non-cellular clots (fig. 6).

The relation, however, of the cellular outgrowths to the nerve fibres was different in the single case in which the neural tube together with its adjoining myotomes were transplanted to the

plasma. In one preparation a piece of tissue, composed of three myotomes and a segment of the dorsal cord of a 62 hour chick embryo, was transplanted to a culture and allowed to grow for four days before it was fixed and stained. The nerves growing from the neural tube were collected into two groups, each of which passed through different intersegmental spaces between the myotomes. A dense mass of cells covered completely all the growing nerves from the neural tube as far as the median border of the muscle plates. The entire picture is similar to that of the segmental nerves as seen in an embryo chick.

The growth of nerve cells aside from the development of nerve fibres has been rarely observed. In one preparation of an isolated piece of neural tube, a single layer of epithelial cells protruded a short distance into the medium. These cells, however, showed no evidence of division. No other evidence of increase in size or thickness of the wall of the tube or outward extension of nerve cells has been noticed. Occasionally in a network of nerve fibres, cells showing characters slightly different from Schwann sheath cells have been observed. Some of these cells, which have wandered out into the plasma, stain deeply and have same general characters of the neuroblasts (fig. 11). However, at present sufficient proof is not available for their exact identity.

MESENCHYMATOUS CELLULAR OUTGROWTHS

The mesodermal tissues studied in this work included muscle of the heart and the myotomes and the interstitial connective tissue cells. The growth of the muscle cells has been noted but rarely and has consisted in the lateral extensions of short chains of striated cells from the myotomes and the heart. The heart muscle cells were further identified by having the same rhythmical contractions as the portion of the heart from which they originated.

The constant appearance and extensive growth in all preparations of the embryonic interstitial cells make them most suitable for the study of cellular activity in these cultures. These cells appear early, growing as a continuous layer over the surface of the tissue or spreading out in various horizontal planes in the

plasma. In the latter they appear as continuous layers, as long chains of cells, or as isolated single cells. The outline of the cell is indefinite during the early periods of active growth, especially in the layers of cells where active division is taking place. They have a pale homogeneous protoplasm filled in part by a single horizontal layer of small, uniform and highly refractile granules. These granules are scattered irregularly throughout the cell in small masses or long rows. The nucleus is well defined from the remaining part of the cell by its great transparency, its freedom from granules and its one or more round or dumb-bell shaped nucleoli, which present a slightly opaque translucence.

The growth of these cells in the plasma consists in a wandering out of cells singly or in small masses from the tissues associated with active division and multiplication. The movement of the cells is very slow. Changes in shape, position and arrangement of granules are only noted by the comparison of repeated observations. The tension of the cell throughout this movement is noticeably undisturbed. The existence of a cellular tension is shown by a constant maintainance of the sharp pointed process, the flat contour and the scattered arrangement of its granules. The association of this tension with the surrounding firm medium becomes evident by suddenly jarring the cell and breaking it free from its fibrin attachments. Such cells immediately assume a spherical form. The granules appear at many levels and completely fill the cell, obscuring the nucleus. The activity, however, is not always lost in these cells. Very quickly, amoeboid movements similar to those seen in leucocytes, are observed. New attachments to the fibrin are made and the broad tense contour is reëstablished. The movements of the cells in this condition of firm attachment to the fibrin of the culture, as seen above, are distinctly different from those seen in leucocytes. A large number of these cells never regained their form but remained inactive, soon showing signs of degeneration. The cells which regained their form were always in contact with the fibrin at some part of their periphery. The importance of this fibrin supporting network is without question from this observation as well as from observations on tissues planted in serum or very fluid clots. These

never showed activity except in a few cases of the latter type.
Growth took place at the few points where the fibrin was in contact
with the tissue. The nerve fibres are also dependent on such
support for their growth, as Harrison has pointed out.

Many of the star-shaped and the irregularly shaped wandering
cells show a constant slow alteration in their outline, while in the
case of the spindle cells the long pointed spindle-form is not changed
often during a passage of several millimeters. The only evidence
of movement in these cells, aside from their change in position,
is the rearrangement of the granules in long rows in the end proc-
esses. In the cases where the cells find attachment along the side
of a coarse horizontal band of fibrin, a movement of the outer
layer of the protoplasm about the axis might account for the ar-
rangement of the granules and the progress forward, with the
maintainance of the cellular tension.

Such growing cells from an isolated piece of tissue have at no
time shown evidence of grouping in a form comparable to organ
formation in the body. Their growth and morphology, as studied
by a careful comparison of the preparations, seem to be governed
by the varied chemical and physical relations of the medium in
which they are grown. To complete the discussion of these cells,
some of the different morphological type will be considered with
their relation to the architecture of the clot.

The spindle cells

These cells grow commonly from the points of the tissue from
which radiate long coarse fibrin bands (fig. 12). They appear as
single slowly moving spindle-shaped cells, closely adherent to the
side of this coarse fibrin band. Aside from the inability to observe
active contractions of this fibrin band, one might conclude that
they had been pulled out of the tissue by some outward force. In
the so-called ring formations observed by Harrison, spindle cells
are formed from preëxisting oval or polygonal-shaped cells by a
visible active change of the clot. The formation of the ring-like
openings occur in the clot after a few days of incubation by a
breaking of the continuity of the net at a point, associated with

contraction of the surrounding fibrin. When this occurs over a growing tissue, the cell layer is broken. The cells become spindle-shaped and form concentric rings about the border of the opening. These cells frequently increase in number. They constantly retain their shape, however, except at the time of active karyokinesis when they assume a rounded form, as may be readily seen in stained preparations.

The question of an active force influencing the growth of these cells is suggested by the long spindle form of the cells growing from the cut ends of a beating heart. In quiescent hearts these cells are polygonal or oval in form. In the specimens where the hearts are transplanted to the same drop of plasma in which a growing mesenchyme tissue is present, the cells of the tissue lying along the line of the transmitted force of the heart beat are seen as long parallel rows of spindle cells.

To aid in the study of the effect of outward force on the cell and the fibrin, a heart was injured on its convex surface. The ends were brought together and held until clotting had taken place. In a few hours the heart was entirely free from the clot except at its cut ends and the opposite injured point on the convex surface. The force of each beat was transmitted directly to the fibrin in a straight line across the drop. After five days, along this line, was a thickened ridge of fibrin composed of long parallel coarse fibrillae. The cellular débris and the small pieces of the tissue had been drawn into it from neighboring parts and arranged in rows parallel to these fibrillae. Preëxisting and growing cells in this zone alike assumed a spindle form. The heart's force was undoubtedly associated with this change in type of fibrin and of cell.

Here, as in the above observations, one can readily associate these cells with this definite fibrin architecture. Whether their form or movement is in any way due to contractions of the fibrin we cannot say, but from the present observations one might assume that through their own activity these cells adapt themselves to the variety of the support at hand.

The irregularly shaped wandering cells

These are apparently preëxisting tissue cells which wander out singly into the plasma. Their contour is variable, oval, triangular, polygonal or star-shaped. They show the characteristic tension and the slow movement of all the mesenchyme. These cells have been frequently associated with clots, or parts of clots, where a small amount of fibrin is present. The fibrin network is made up of very delicate fibrillae, surrounding large open spaces. The more extensive wandering of mesenchyme cells has always been associated with this type of fibrin architecture. Wandering is characteristic of all these cells, but it is considerably limited in most of the preparations. Another type of cell to be considered at this time grows from a tissue deeply and firmly embedded in a dense clot. These are elongated masses of protoplasm which send out many long cone-shaped processes from their end or along their borders. They grow very slowly, never reaching more than four cell lengths. The boundaries of the individual cells are not as a rule clear and they appear as large multinuclear cells. The progress of cellular growth is apparently hindered by the density of the clot (fig. 13)

Mesenchyme cells growing in layers

These cells comprise a group where growth and multiplication are most evident. Their frequent location is on the lower surface of the clot or along the wall of the large open concavities, which appear in the older preparations. The cells appear as closely adherent, definitely or indefinitely outlined cells spreading out in a continuous layer from various parts of the tissues. They vary from an oval (fig. 12) to a polygonal form, sometimes approaching a spindle shape. The development of this layer is due in part to a wandering out of the preëxisting tissue cells associated with active multiplication by mitotic division. In the early periods of growth or in very thin clots (fig. 3) a single layer of these cells is noticed, but after the fourth or fifth day in deeper clots, a layer two or three cells thick has often formed.

Associated with this type of cellular growth is a constant thinning and often a complete disappearance of the clot over the thicker layer of the cells (fig. 3). Following this disappearance of the fibrin a large drop of fluid collects on the lower surface of the drop. This fluid is much in excess of the serum which could be squeezed from a clot of plasma of the same size. Its formation is probably associated with the cellular growth. At these points where fibrin has disappeared, the cells are most active, karyokinesis being common (fig. 14).

Actual division of the cells has never been completely followed under the microscope. In the stained specimen, however, all stages of mitotic division can be readily found in preparations under six days old. The shape of the cell, as especially studied in the spindle types, does not change during the prophase; at the period of metaphase, however, it becomes round and remains in this shape until the daughter cells are formed. Division of the nucleus of a direct type has been seen only in giant cell formation, which is common after the sixth day of growth. Active mitotic division may continue for several days. I have seen many mitotic figures in a few of the specimens fixed at the eighth day of cultivation.

The study of the mesenchyme cells has demonstrated that the plasmatic medium has power of preserving the life of the cell as well as supplying sufficient nutriment for growth, as shown by the active division of the cells.

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CONCLUSIONS

1.   The tissues of the embryo chick can be cultivated outside the body in a medium of plasma, prepared from blood of adult chickens.

2.   Nerve fibres grow from the embryonic neural tube which has been cultivated in blood plasma. These fibres react to specific nerve stains and have the histological characters of normal embryonic nerves.

3.   The growing nerves can be readily observed in these cultures. They grow by an independent activity of their own protoplasm.

4.   The growth of the mesenchyme tissue consists in the wandering out of the preëxisting tissue cells and their multiplication by mitotic division.

5.   The morphology and the arrangement of the mesenchyme cells are dependent on the physical characteristics of the plasma clot.

6.   Muscle cells appear as chains of striated cells from the border of the heart and the myotomes.

7.   The embryonic heart transplanted to this culture medium beats for several days, often with a normal rhythm and force.

8.   This method of growing tissues in culture permits only of the histogenetic study of the cells and the nerve fibres. Structures comparable to organ formations of the body are never observed.

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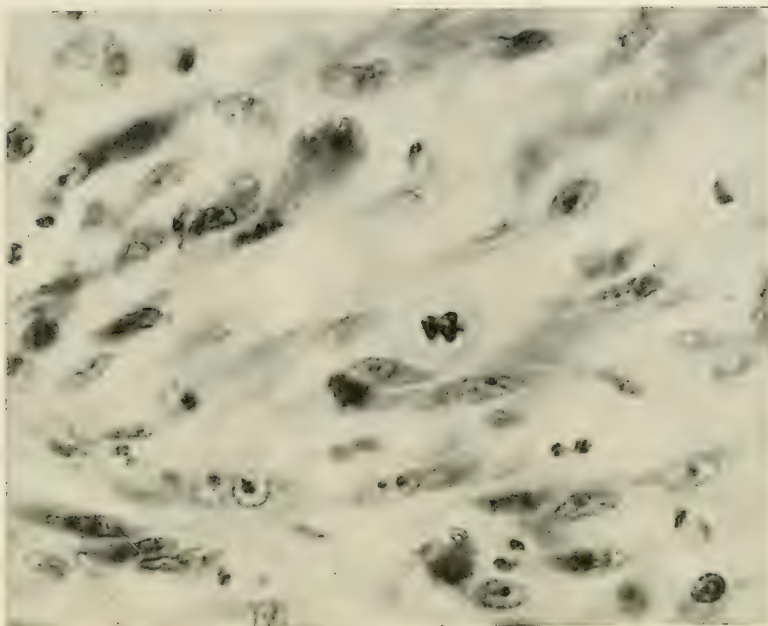
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PLATE 2

EXPLANATION OF FIGURE

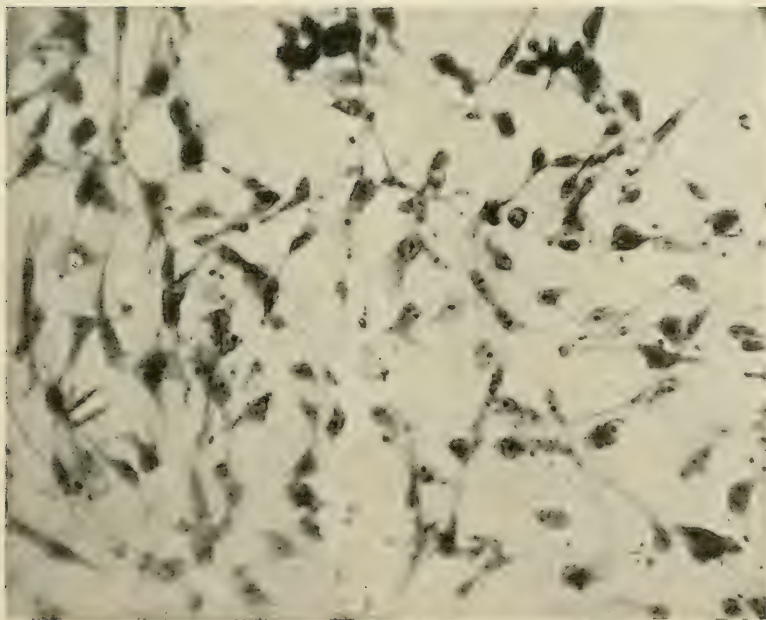4   An isolated piece of neural tube with nerve fibres growing in the clot.   Stained in Held's molybdic acid haematoxylin.   ×325.

4

*Camera lucida drawings of the stained preparations*

EXPLANATION OF FIGURES
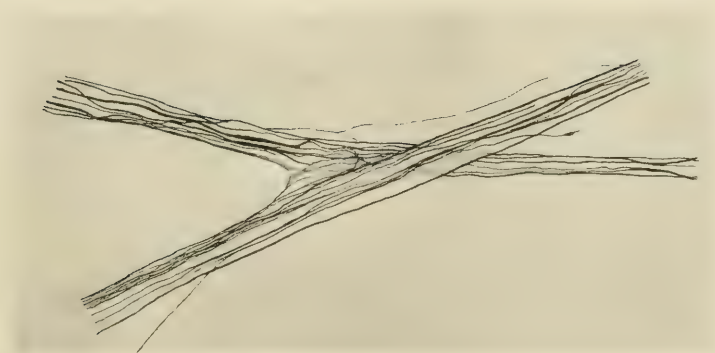
5 Nerve endings drawn from nerves pictured in fig. 4. ×725.

6 Nerve ending in a fibrin network near a large mesenchyme giant cell. Five-day culture. Stained in Held's molybdic acid haematoxylin. ×725.

7 Anastomosing nerves which have grown on the surface of the clot. Five-day culture. Stained in Held's molybdic haematoxylin. ×525.

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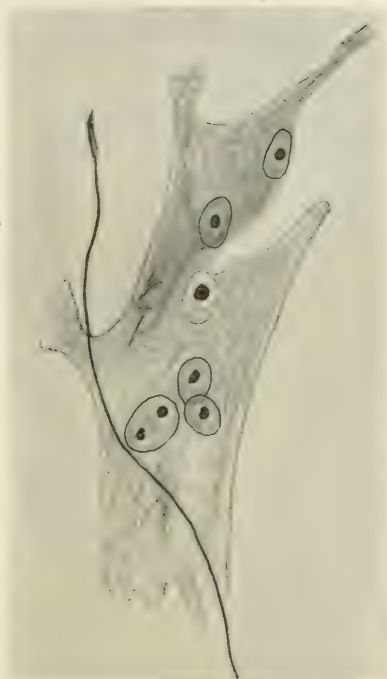
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PLATE 4

Photographs of the preparations stained in Held's molybdic acid haematoxylin

EXPLANATION OF FIGURES

8   Nerve fibres growing from a group of ten nerve cells. Four-day culture. ×144.

9   Nerve ending in a fibrin network. Five-day culture. ×1320.

10· An enlarged photograph of one of the nerve ends shown in fig. 8.   ×540.

11   An isolated cell lying over a network of nerve fibres. Cell stains very deeply. Nerve fibres apparently arising from two pales of this cell. Five-day culture. ×324.

Camera lucida drawings of the cells

EXPLANATION OF FIGURES

12   Spindle-shaped mesenchyme cells associated with long radiating bands of fibrin.   Oval cells of a type similar to those growing in lower surface of the clot are seen near the tissue.   Early signs of degeneration are seen in the appearance of the large and very refractile granules in many of the cells.   Ten-day culture. Drawn from the living cells.   ×300.

13   A large multi-nuclear cell growing from the tissue into a dense clot.   Five-day culture.   Drawn from the living cell.   Tissue schematic.   ×350.

14   Cells growing in a layer on the lower surface of the clot.   Four-day culture. Stained in haematoxylin and Congo-red.   ×725.

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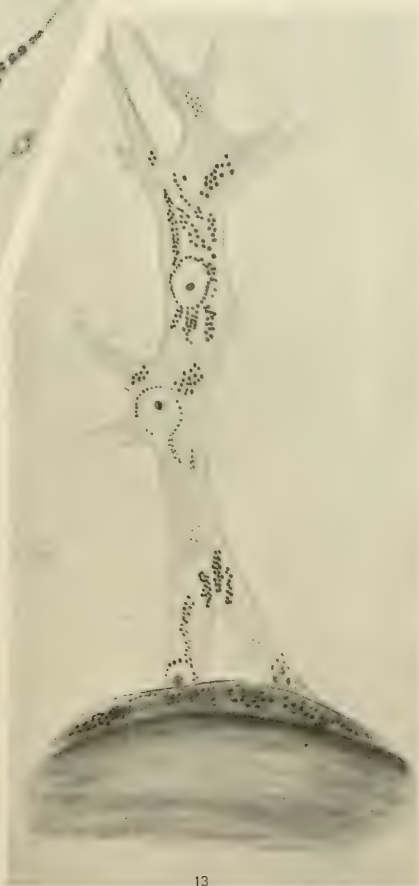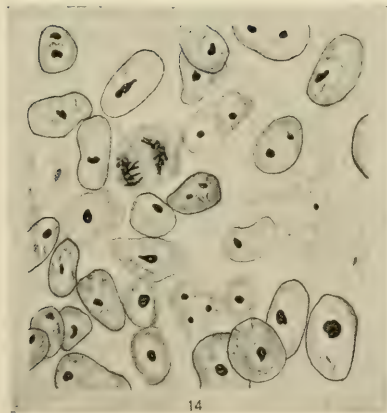
A STUDY OF THE DIFFERENTIATION OF NEURO-BLASTS IN ARTIFICIAL CULTURE MEDIA[1]

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TEN FIGURES

Few biological problems offer greater difficulties than those that attempt to deal concretely with the factors involved in the process of differentiation. In the light of our present knowledge, as well as on purely *a priori* grounds, the two extreme views, that of Driesch and his followers, that "the prospective value of a blastomere is a function of its position," and the mosaic theory in its most pronounced form, that development "is to be regarded as a mosaic work of self-differentiating cells,"[2] seem equally untenable. The work of numerous investigators on a great variety of forms has established beyond question that when certain oöplasmic substances are destroyed the tissues into which they normally develop are lacking. On the other hand the fact that development may proceed for a time after the destruction of portions of the organism does not, for many reasons, demonstrate that the remaining portions are in any degree self-differentiating. First of all it is noticeable that development after the injury depends not so much on the extent as on the character of the substances destroyed, For example, Conklin,[3] in the study of ascidian eggs, found that a right or left half embyro develops much farther and much more normally than an anterior or posterior half, and from

[2] Wilson, E. B., '04. Mosaic Development in the Annelid Egg. Science, vol. 20.
[3] Conklin, E. G., '05. Mosaic Development in Ascidian Eggs. Jour. Exp. Zoöl., vol. 2.

the description of the experiments it is evident that this is due to the fact that part of all the oöplasmic substances are still left in the first case, while in the second, all of some of these are removed. In the right or left half, therefore, all the metabolic processes characteristic of the complete organism may go on, with the possibility of all the normal inter-reactions, while in the anterior or posterior half some of these are omitted.

In the development of the form of any animal, the location of different materials must play an important part, but in the differentiation of any particular kind of tissue the presence, somewhere within it, of each material normally found, is, I believe, much more important. To put it in another way, an ovum with, for example, six kinds of oöplasmic substances may be compared to six beakers with permeable walls each containing one or more chemical compounds, and all immersed in the same pan of water. If beaker 1 is placed next to beaker 6, the sum total of the final products will be likely to vary, at least in quantity, from those that would have been obtained if it had been placed next to beaker 2. But if the products or the reactions going on in beaker 1 have any chemical affinity for the substances in beaker 2 these will doubtless, eventually be affected thereby.

It is because of this that the transplantation of embryonic tissue to abnormal positions in the body may prove nothing concerning the question of correlative or self-differentiation. As I have tried to show in a previous paper,[4] the development of nerve fibres and muscle tissue from a portion of the medullary canal transplanted with somites attached may be due to the presence in the circulating medium of this region of some substance found in its normal location. And I know of no experiment in which the wholly normal differentiation of any tissue has occurred, in which this possibility, absolutely essential for demonstrating self-differentiation, has been eliminated.

But aside from the fact that experimental evidence has failed to establish that some stimulus outside the tissue itself may not

[4] Shorey, M. L.,'09. The Effect of the Destruction of Peripheral Areas on the Differentiation of Neuroblasts. Jour. Exp. Zoöl., vol. 7.

have activated its development, there are strong logical grounds against such an hypothesis. If tissues are self-differentiating, it is impossible to account for their interdependence in the adult without postulating a physiological discontinuity in the life of the individual cells. In this connection the relation between nerves and muscles will serve as an illustration. It is well known that the life of a nerve can not be maintained after the extirpation of its end organ; and if it can not be, then it must be due to its inability to carry on its normal metabolic processes. If it can do this independently during development, then there must come a point at which embryonic physiological processes are checked and adult processes begin. To avoid this difficulty a functional and a pre-functional period have been postulated, but since the functions which the cell performs for the body as a whole are merely incidental to the processes necessary for its own existence, it is evident that if it performs different offices at different times, it must be due solely to the fact that its metabolism differs. The cell once differentiated goes on building up protoplasm of the same architectural pattern during the rest of the life of the organism, therefore, the point at which it begins to assume the offices which it performs for the adult must begin at the beginning of differentiation and not at the end. Any cell which, in the adult organism, had no physiological inter-relation with some other cell would be a foreign body, not an organic part; if the forces acting in its differentiation are not identical with those acting in its physiological relations, then the structure established at the time of differentiation could not be maintained.

In view of the above considerations, I would suggest as a working hypothesis, that the development of different kinds of tissue may be regarded as the result of a chain of chemical reactions and inter-reactions between oöplasmic or organ-forming substances, and that any given tissue will be formed only when a given primordium is acted on by a definite force external to itself; and while pressure, contact, and forces external to the organism, may play a part, *one of the sources of stimulation will always be found to be the metabolic products of other tissues.*

How may the truth of such an hypothesis be tested?

As stated above, the necessity of a given primordium is well established, but for each tissue we must still ask, Is some external stimulus necessary to produce differentiation? If so, what is its nature and source?

For certain specific structures these questions may be answered, at least in part, by experiments in regeneration or transplantation, as has been done by Herbst in the study of the eye of the crab and by Lewis in the study of the eye of the frog. But for tissues which are not restricted to very limited areas, it is evident, that neither of these methods can be effective. Nor can absolutely crucial results be obtained by the removal, in forms in which no regeneration occurs, of the primordium of some organ known to be closely related in the adult to another; for such experiments are always open to the objection that any abnormalities are purely traumatic effects, an inhibitory substance being, perhaps, produced by the wound. My own attempts to establish the necessity of the presence of muscular end organs for the development of motor nerves are a good example of this. From a most careful study of all the conditions I am convinced that the effects of the wound do not enter into the results, but I can give no conclusive evidence that this is so.

Therefore, when in 1907,[5] Harrison published an account of the development of nerve cells removed from the body of a frog and placed in a drop of lymph, a distinct advance in method was made, at least theoretically. And I shall describe in this paper a few out of a great number of experiments in which I applied this method to a further study of the differentiation of neurones. From the development of neuroblasts into nerve cells and fibers in lymph Harrison concluded that they are self-differentiating, while from the experiments in which I demonstrated that when the primordium of given muscles is removed most of the motor cells which normally innervate them fail to develop, I concluded that the motor neurones are in some way dependent on the muscles for differentiation. The only hypothesis by which these two lines of experiment can

[5] Harrison, R. G., '07. Observations on the Living Developing Nerve Fiber. Ant. Rec., vol. 1.

be harmonized is that it is the metabolic products of muscles which act as a stimulus. My previous work furnished no experimental evidence regarding the means by which the muscles influence the nerves, while Harrison's experiments are open to the objection that in the lymph the products of muscular activity might, and almost certainly would, be present.

In order to test the truth of my hypothesis by this method, both positive and negative results must be obtained. That is, a medium must be found in which neuroblasts remain alive and active, but do not develop nerve fibres, while if some substance or substances produced by the activity of muscles is added, fibres do develop.

I first attempted experiments with portions of the medullary canal of the chick, but these results were always negative, with two possible exceptions. Pieces of the canal or isolated neuroblasts may be kept alive for days in a variety of culture media. White of egg, gelatin solutions containing peptone or beef extract, solutions obtained by allowing muscular tissue from chicks or adult fowls to stand for a few hours in normal salt solution, lymph from a number of different kinds of animals, if kept without infection, will all serve as nutrient material for a period of from three to at least fourteen days. That the cells are alive can not be doubted, for after a few careful observations, one can not fail to recognize evidences of degeneration if it is present. I can not affirm with certainty that division ever occurs under these conditions, for while I have occasionally seen contiguous cells which from all appearances, were just completing the process of division, I have never seen it in sufficiently early stages to be convinced.

But while in hundreds of cases the neuroblasts have remained in a vigorous condition for days after fibres would normally develop, on only two occasions, as stated above, has there been any indication of this. As no growth was seen after what may possibly have been nerve fibers were first observed, and as no method of staining was attemptd with these, and numerous repetitions of the same conditions failed to produce the same results, I am still very skeptical regarding them. It should perhaps, be added, that in both of these cases extracts from muscles were present.

Experiments with neuroblasts from Necturus were more satisfactory, although I have failed to repeat successfully Professor Harrison's experiment of developing nerve fibers in a drop of lymph from the adult.

The culture medium with which I obtained successful results contained the following ingredients:

4000 cc. tap water;   12 gm. beef extract;
  40 gm. peptone;   560 gm. gelatin.
  20 gm. NaCl;

This was neutralized with NaOH, and, after cooking, 40 cc. of 1 per cent. HCl were added. Drop cultures were made by adding either isolated cells or small portions of the medullary canal to this. When placed in this medium the isolated cells were oval in shape, with a perfectly even contour. Figs. 1 and 2 represent two typical cells about forty-three hours later. These two cells were examined at intervals for the next twenty-four hours, but there was no appreciable change. From a small piece of the medullary canal on the same slide, a mass of fibres were developing on one side, and a scratch on the cover glass just beyond the end of the longest fibre was noted, and used as a means of determining the extent of growth. Twenty-four hours later this fiber had crossed the scratch and grown for some distance beyond; the other fibres had also increased in length. Fig. 3 represents the mass of fibers at this time, the cells from which they arise not being shown.

To determine with certainty that these could not be lines of coagulation or any other similar phenomena, a dilute solution of methylene blue was added and allowed to stand for a few moments. On removing it the gelatin remained colorless but the cells and fibers were distinctly stained, and that the latter were outgrowths of the former could not be doubted.

The experiment was repeated, and although not always successful, on two slides, I obtained many short fibres and a few longer ones, with the characteristic change in the shape of the cells. A few of these are represented in figs. 4 to 10. In all cases, after a careful examination without staining, methylene blue was added
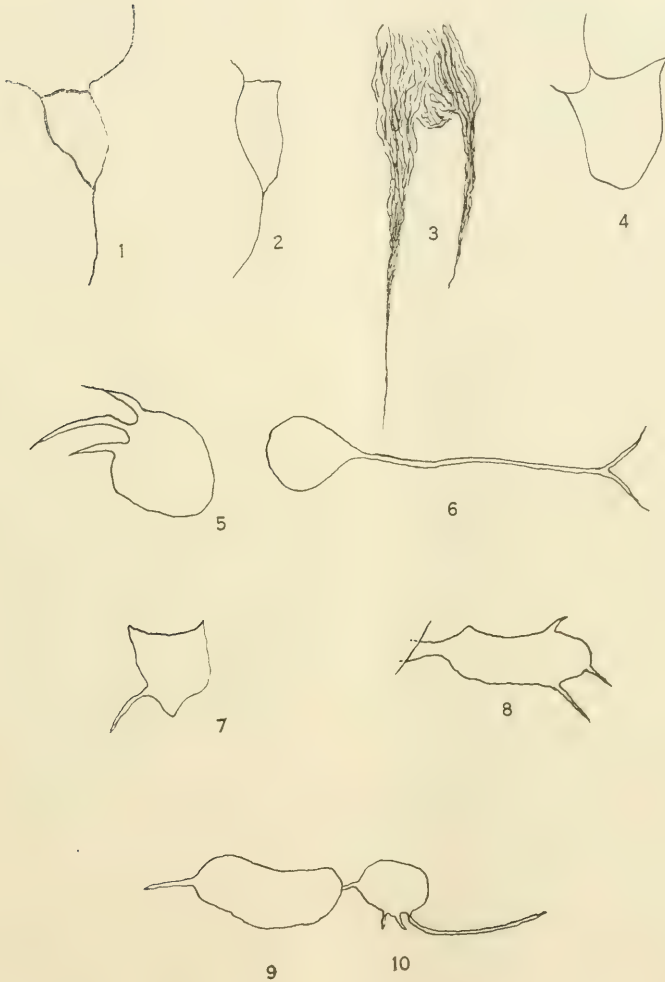
Figs. 1 and 2, and 4 to 10 were drawn after isolated, wholly undifferentiated, neuroblasts from necturus had developed from 40 to 70 hours in the culture medium described. Magnification about 400 diameters. One end of a fibre represented in fig. 8 grew beneath an opaque mass, and a portion of one represented by fig. 10 grew beneath 9.

Fig. 3 represents a mass of fibres growing from a small portion of the medullary canal about 67 hours after being placed in the culture medium.

For further explanations see text.

with the result as given above. Repetitions of this experiment could not be continued because it soon became impossible to obtain Necturus with undifferentiated neuroblasts.

Regarding the fibers that develop, it might be added, that instead of one long process, with, perhaps a number of much shorter protoplasmic outgrowths, characteristic of motor cells of the spinal cord, there are often a number of processes of equal length. A possible explanation of this is that in the embyro the movements of the lymph are such that a greater stimulus reaches the cell at the point where the longest fiber forms, while in the culture medium the substances which act as a stimulus are equally diffused. Occasionally swellings were noted at the ends of the fibers, as Harrison has described for the frog, but these were not characteristic.

At the same time a culture medium made according to the same formula as the one used above, with the beef extract omitted, was prepared, and drop cultures with neuroblasts from the same embryos were made. But although many of these were prepared and examined, more than of those containing beef extract, and the cells were still in a vigorous condition at the end of two weeks, no development of fibers, and no change in the shape of the cells could be observed.

Do these facts justify a belief that the neuroblasts are stimulated to differentiate through the presence of the metabolic products of muscular end organs? Of themselves they do not furnish crucial evidence, since in many instances differentiation does not occur even in the presence of these products. This may, however, be accounted for by considering the condition of the cells at the time when they are placed in the culture medium. It is probable that the the change in metabolism, from the completely undifferentiated to the adult cells, is a gradual one in both nerves and muscles, and it is quite possible that the two keep pace, and that a neuroblast must reach a certain stage of development before it can be stimulated by the metabolic products of *adult* muscles. The fact that in all cases in which I have been successful in producing nerve fibers the neuroblasts were taken

from the medullary canal in a comparatively late stage of development lends a certain amount of support to this suggestion.

A greater objection is found in the fact that with a commercial extract of muscle, or indeed in any extract, many substances might be introduced which are not specifically the products of muscular metabolism. No attempt was made to discover what substances in the beef extract were the source of the stimulus, and, therefore, as far as these experiments alone are concerned, it might equally as well be some inorganic salt which may be found in it. But taken in connection with the fact that when the primordium of a muscle is destroyed, development of the nerves does not occur, it is certainly added testimony for the belief that the products of muscular metabolism are the specific source.

But if there may still be some doubt regarding the source of the stimulus, the present experiments have, I believe, confirmed the conclusion previously reached that neuroblasts are not self-differentiating. For the possiblity of the presence of a specific inhibitory substance in all the varieties of indifferent media used can hardly be maintained.

REGENERATION AND CELL DIVISION IN URONYCHIA

GARY N. CALKINS

FIFTEEN FIGURES

CONTENTS

Regeneration in Protozoa has been more or less exhaustively studied by different observers, and Balbiani, Hofer, Gruber, Nussbaum, Verworn and others have contributed not a little to our knowledge of the physiology of the cell through such experimental work. So far as I am aware, however, no one has studied the regeneration power in relation to cell division, and it is generally assumed that a protozoön is able to regenerate at all times with equal facility.

The experiments described in the following pages were made during the summer of 1910 at the biological station at Roscoff, Finistère, and I take this opportunity to express my grateful thanks to Professor Yves Delage for the many courtesies shown me while in his laboratory.

MATERIAL AND METHOD

After many attempts to find a form sufficiently large and hardy for successful experimental merotomy, Uronychia transfuga Stein was finally selected.   This is one of the hypotrichous ciliates belonging to the family Euplotidae.   The Roscoff form differs in some minor respects from the species described by Stein, but as the organism is somewhat polymorphic and shows such wide differences in size and appearance, I would not venture to make a new species.   The band-form nucleus characteristic of the family is here replaced by a chain of large nuclear spheres during the vegetative stages, but these coalesce during division to form the typical band.   The organism is somewhat longer than broad, with bluntly rounded anterior and posterior ends, and is elliptical in shape.   The hardened ectoplasm forms a practical cuirass about the cell and gives to the latter much of its durability.   The dorsal surface is slightly arched and sculptured on the anterior margin for the insertion of the membranelles of the adoral zone, while posteriorly it is deeply cut out for the posterior cirri.   The latter are separated into two groups by a posterior process or lobe of the dorsal surface (fig. 1).   To the left of this lobe originate two large and one small cirri, one of the larger cirri being dorsal to the other large one.   On the right side of the lobe are seven cirri of variable size.   Three of these are inserted dorsally and are sharply curved, sickle-like towards the left.   The bases of all three of these are in a line and the extreme left one lies partly on the dorsal lobe.   A similar set of three cirri originate on the ventral surface, opposite the dorsal set, but unlike the latter, the cirri are straight or but slightly curved.   One large cirrus, also curved sickle-form to the left, arises between the two sets of three and on the extreme right edge.   Two smaller cirri arise on the right margin and one or two small ones originate on the ventral surface (fig. 1).

The adoral zone of Uronychia consists of large membranelles running from the anterior right margin, around the anterior margin and then ventrally in a broad curve to the mouth.   The membranelles are inserted in the serrations of the anterior mar-

gin. The peristome is a relatively deep, sunken area covered over by a thin ectoplasmic sheath. An undulating membrane endoral membrane E), which on occasions may be everted, balloon-like to the outside, lies on the left side of the peristome, and a similar, pre-oral, membrane lies on the right (P). A few small cirri are inserted at the base of the undulating membrane and near the mouth, but they are inconspicuous and difficult to see.
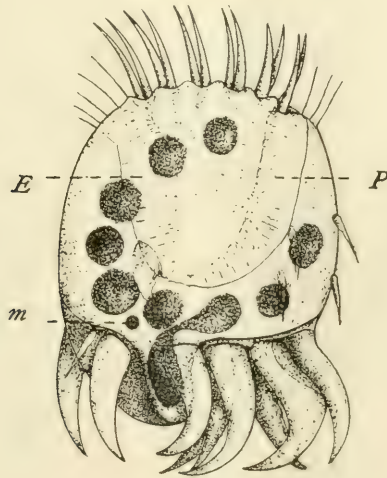
Fig. 1 Uronychia transfuga Stein drawn from life for cirri, adoral zone and peristome, from dorsal side. E, endoral membrane; P, preoral membrane; m, micronucleus. The lowest macronuclear fragment lies in the dorsal lobe.

The macronucleus consists of from 10 to 14 large spherical bodies of chromatin, arranged as in the figure to form a broken horse-shoe. One of the masses of chromatin is always in the posterior lobe of the dorsal surface. The micronucleus (m) is single and much smaller than any of the bodies of chromatin of the macronucleus. It usually lies between two of the more posterior masses of macronuclear material.

Movements. There are two distinct types of movement, one, a steady or uniform movement in the direction of the anterior

end, the other a vigorous backward leaping movement due to the
giant cirri. When irritated the organism gives a backward leap
with one or a few of the posterior cirri, moving only two or three
times the length of the body; this is repeated for three times as a
rule, and then, if the irritation continues, all of the caudal cirri
are brought into play and the cell is swept away backwards like
a flash. It darts back and forth across the watch glass for several
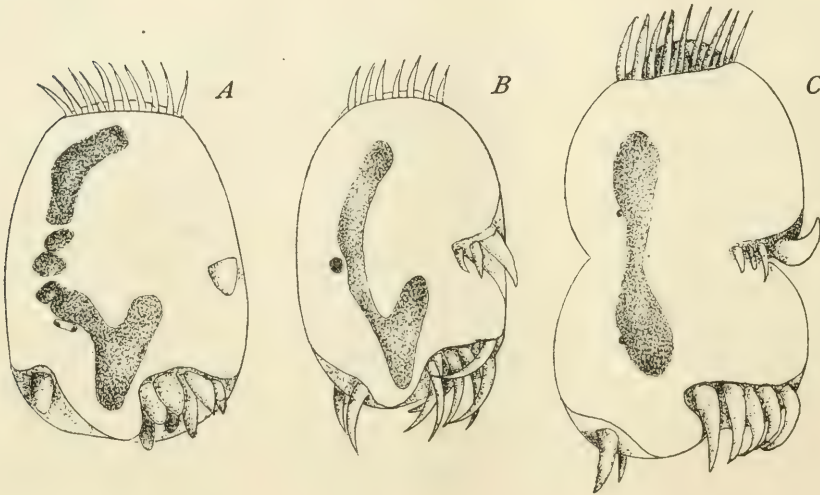
Fig. 2 Stages in the normal cell division of Uronychia (camera drawings from
preparations). *A*, later stage in the condensation of the macronucleus, three
spheres not yet fused. The micronucleus is in the end phase of division. A new
cirrus is forming in the regeneration zone of the anterior organism, and new cirri
have replaced the old ones of the posterior end. *B*, *C*, later stages in division.

seconds, finally coming to rest with a jerk and moving off at once
in the opposite direction. Its movements are strikingly sug-
gestive of a bird's flight and manner of alighting.

Division. Under the cultural conditions of the laboratory,
Uronychia divides about once in 36 hours. The process is com-
plicated and after the first visible evidence of division, requires
about 6 to 8 hours for completion. As Wallengren ('01) showed

for this and for other hypotrichous ciliates, all of the old cirri are absorbed and new growths take their places. The new caudal cirri for the anterior cell are precociously formed, the three dorsal sickle-formed ones appearing first the others not appearing until the division is well advanced (fig. 2). New membranelles are similarly formed for both halves and a shifting of the axes of the two connected cells gives ample room for the further development of these appendages before complete separation (fig. 3).
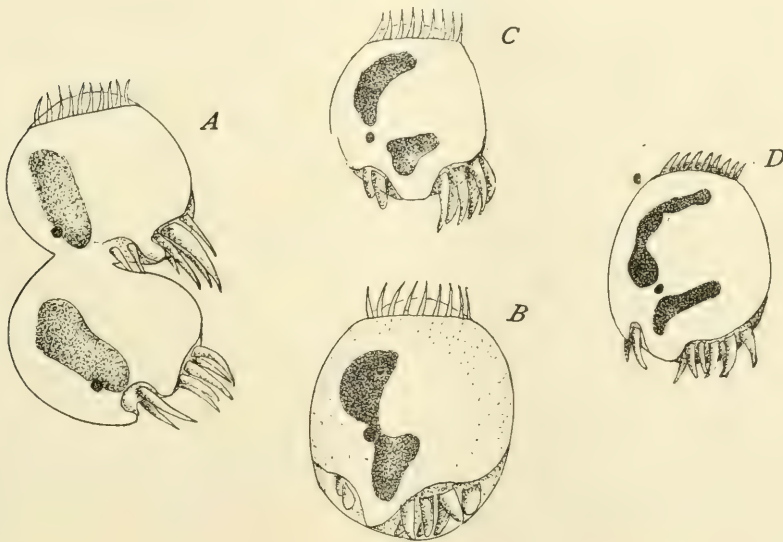
Fig. 3   Final stages in the division of Uronychia (camera drawings from preparations). A, a late phase before separation; B, a cell immediately after division; C, a cell fifteen minutes after division; D, a cell one hour after division.

During these precocious formations, the nucleus gradually prepares for division. The first step is the fusion of the large masses of chromatin into a single band-formed nucleus (fig. 2, A, B). This fusion is not completed until after the new cirri are well advanced in growth. The micronucleus is very minute and partly embedded in the macronucleus, nevertheless it divides by mitosis, a minute spindle being formed. The macronucleus

on the other hand divides without mitosis, and shortly after
division it breaks up into small spherical or ellipsoidal chromatin
masses typical of the resting or vegetative stages.   Stages in
this re-organization of the resting nucleus are shown in fig. 3.
The first fragment formed after division becomes the nuclear
granule of the posterior lobe while the micronucleus lies between
the first two fragments.   These first two parts next divide into
two, the four into eight; some divide again, while others do not,
the result being from 10 to 14 macronuclear fragments in the vege-
tative stages.   These nuclear relations are so characteristic that
any deviation means an irregularity or abnormality, and they are
particularly important in connection with the orientation of frag-
ments.

EXPERIMENTAL

For cutting Uronychia a small, fine-pointed, carefully sharp-
ened scalpel was used.   A serrated or "saw edge" was always
avoided.   The cell to be operated was first isolated in a small
drop of water on a glass slide and then cut under a microscope
it was found that, after some practice, a cell could be cut without
mangling and in any desired plane.   The cut parts were then iso-
lated in a few drops of the original medium; these were then cov-
ered by a glass coverslip supported on thick glass feet, and the
whole put away in a moist chamber.   In this way Uronychia after
operation may be kept for a week or ten days or longer if necessary,
normal forms dividing regularly under similar conditions.   As
a rule the experimental forms were not kept more than four
days, and frequently only until regeneration was complete.   The
fragments isolated were always carefully studied and sketched
to show where and how the knife had passed through.   For fix-
ing sublimate acetic was used, and for staining the best results
were obtained with Delafield's hematoxylin.

1.   *Uronychia cells cut immediately after division*

If the cell is cut immediately after division and before the nucleus begins to fragment into the normal condition, there is but little power of regeneration and then only when both nuclei are present.   In some cases there is no regeneration at all.

Experiment 28.   Both cells were cut at once after division; the anterior cell was cut longitudinally and in such a plane that no nuclear material was included in one of the fragments. This failed to regenerate, but the other fragment formed a perfect cell

4 A   **4 B**
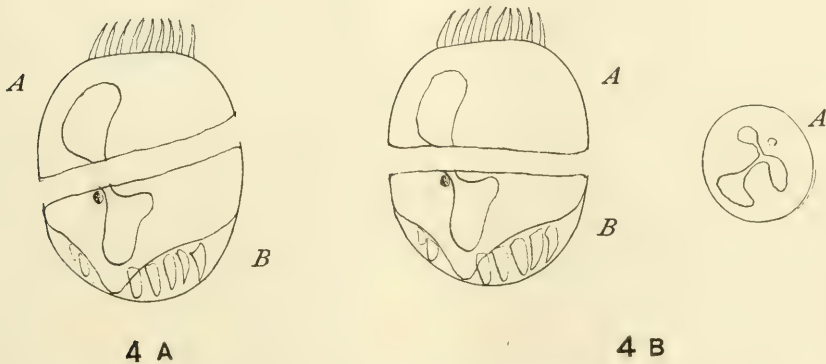
Fig. 4*A* Experiment 42. The cell, immediately after division, was cut obliquely as shown. Neither fragment regenerated although the sister cell divided twice in the next three days. 4*B*. Experiment 28. The cell was cut immediately after division. *B* regenerated perfectly, *A* failed to regenerate although the nucleus underwent the first stages of reorganization (*A*, on right).

within 24 hours, normal in size and form.   The posterior cell was cut transversely as shown in the diagram (fig. 4*B*).   The macronucleus was almost equally divided between the two fragments, the micronucleus was retained in the posterior fragment. The latter regenerated perfectly within 24 hours, the anterior fragment failed to regenerate although it continued to live for 48 hours when it was killed for the determination of the nuclear structure.

Experiment 36.   Here the plane of the cut was longitudinal. Macronuclear material was retained in each fragment, while the

micronucleus was present in only one. The latter regenerated perfectly in 24 hours, the former not at all, although it continued to live for three days.

Experiment 39. The cell was cut transversely as in fig. 5, the macronucleus being almost equally divided. The fragments were kept under observation for three days, the micronucleus holding fragment (B) regenerating in 24 hours, the other not at all.

Experiments 42 and 43. Here the cells were cut obliquely through the anterior region (fig. 4A,) and neither fragment
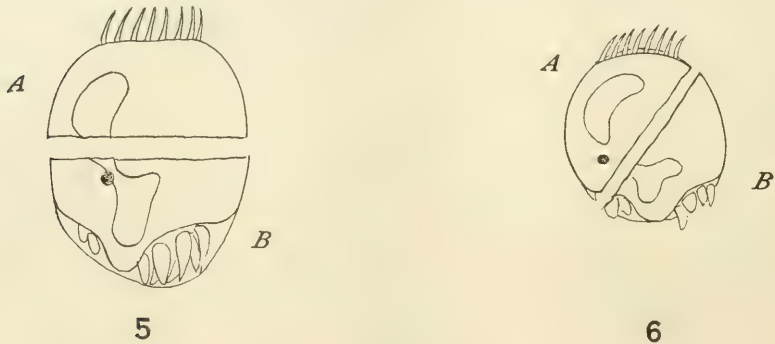
Fig. 5 Experiment 39. The cell was cut immediately after division as shown. Fragment *B* regenerated in 24 hours; fragment *A* not at all.

Fig. 6 Experiment 51. The cell was cut one hour after division. *A* regenerated perfectly in 24 hours, *B* lived for 72 hours and died without regenerating.

regenerated in the three days of observation, while the sister cells kept for control divided twice in the same time.

Experiments 49, 57, and 61 gave results similar to those of nos. 28, 36 and 39, the micronucleus holding fragment regenerating perfectly, the other not at all.

2. *Cells cut from fifteen minutes to one hour after division*

The power to regenerate is no better developed from fifteen minutes to one hour after division than it is immediately after.

Experiments 45, 46, 48, 49 and 51. In all of these cases the cells were cut from fifteen minutes to one hour after division.

At this time the macronucleus is divided into two nearly equal parts (cf. fig. 3, *C*). In all cases the micronucleus-holding fragment regenerated, while the other did not (fig. 6).

These thirteen experiments gave no single case of double regeneration of the cut fragments, while in two cases neither fragment regenerated. This fact is significant in view of the supposedly high growth energy of young cells.
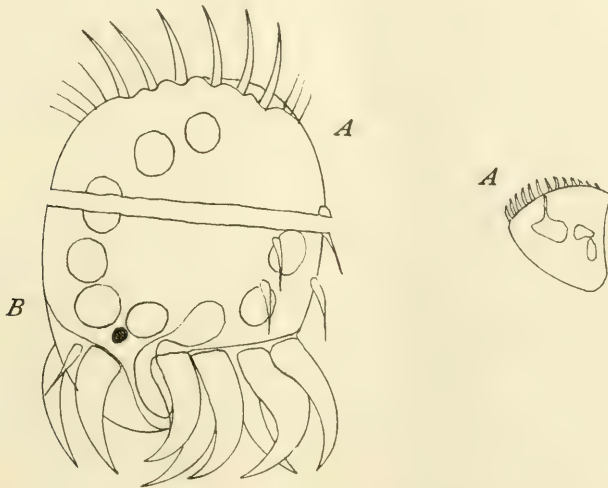
Fig. 7 Experiment 34. Cell about eight hours after division. *A* failed to regenerate but lived for more than 72 hours (see *A* on right). *B* regenerated in 24 hours.

3. Cells cut from 8 to 16 hours after division, i.e. about midway between two division periods

In fragments of cells cut at times about midway between division periods both the power of regeneration and the rate of regeneration are greater than immediately after division. In the later periods fragments may regenerate without the presence of a micronucleus.

Experiments 8, 15, 20, 23, 24, 31, 32, 33, 34, and 38 were based upon cells cut from 8 to 16 hours after division. In nos. 8 and 24 one fragment was lost in each case, but the other fragments regenerated perfectly. In nos. 15, 23, 31, 32, 33, 34, and 38 there

was complete regeneration of the micronucleus-holding fragment, while the other fragment failed to regenerate. Experiment 34 is a typical case of this group. Here the cell was cut almost through the center as shown in fig. 7. The anterior part (A), without a micronucleus, did not regenerate and was killed after 72 hours (see fig. 7A); the posterior part (B) regenerated perfectly in 24 hours.
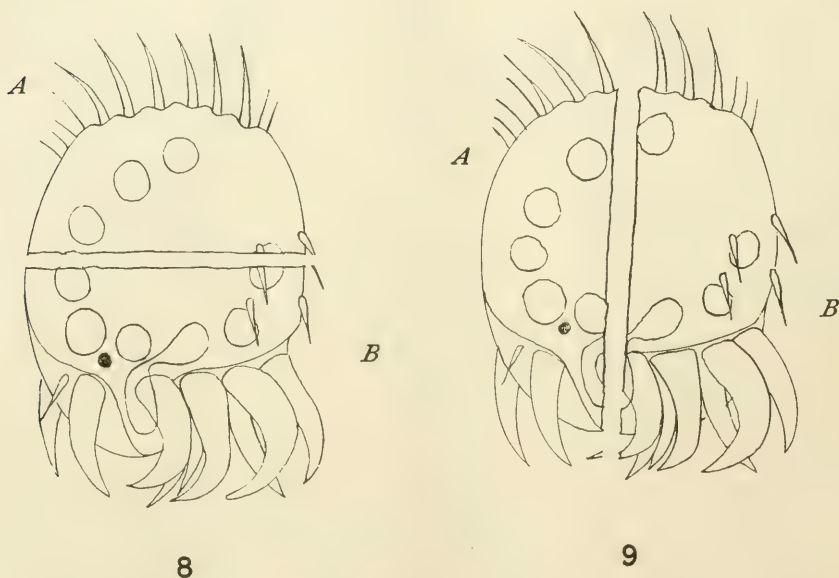
Fig. 8 Experiment 21. Cell from 24 to 30 hours old. Both fragments regenerated perfectly in 24 hours, although A was one-quarter size.

Fig. 9 Experiment 37. Cell from 6 to 8 hours old. Both fragments regenerated in 24 hours. B, however, did not grow but died the following day.

Experiments 21 and 37. Here the cells were cut as above but both fragments in each case regenerated into perfect organisms. In no. 21 the cell was 24 hours old when cut through the center as shown in fig. 8; after one hour A showed no sign of regeneration but B had replaced 4 membranelles; after 2 hours A was still not regenerating, but B had 6 membranelles. Finally, after 24 hours, A was completely regenerated but only of one-quarter size, while B was full size and normal. After 48 hours both were large and

normal but after 72 hours A had one abnormal cirrus; this how-
ever was only temporary, the cell being entirely normal the fol-
lowing day. B divided on the third day but one of the products
was only quarter size and both died the following day.

In experiment 37 the cell was cut longitudinally as shown in
fig. 9, and from 6 to 8 hours after division.   After 24 hours A
was large, normal and active; B was also normal and active but
small and died after 48 hours.

In cells midway between division periods therefore, the power
of regeneration is much better developed than in cells immediately
after division.

4.   Cells cut during the process of division

*A.   In the early stages and before the compact dividing nucleus
is formed.*

*If the cut is transverse or oblique and not directly through the cen-
ter, the larger fragment with the micronucleus divides in the original
plane.   All three fragments regenerate into normal organisms one
of which has no micronucleus.*

Experiments 9, 11, 12 and 13, were cells cut transversely after
the first external traces of division were seen.   At this period
the nucleus is still distributed, (cf. fig. 2) but new cirri are form-
ing in the anterior half and at the bases of the old cirri in the pos-
terior half.   Experiment 12 is typical.   Here the cell was cut
above the mid line as shown in fig. 10.   After 3 hours A had not
begun to regenerate, while B was dividing in the original central
line of the cell.   After 18 hours however, A was completely regen-
erated although somewhat abnormal in shape. B had divided
forming one large and normal cell B' and a much smaller quarter
size cell, B''.   After 42 hours A was somewhat smaller but per-
fect and very active, B' was large and perfect; B'' as before.
After 66 hours A was still smaller and emaciated B' had divided
while B'' remained as before.   Finally, after 90 hours, A and B''
died.   Experiment 14 gave exactly similar results.   In experi-
ments 9 and 11, the cuts were almost directly through the plane
of future cleavage.   In no. 9 only two cells were formed; in

no. 11 a small bud was formed in addition but this bud did not develop.

In experiments 16, 25 and 75, the cells were cut obliquely, no. 75 being a typical case.   Here the cut passed through the posterior end of the cell as shown in fig. 11.   After 18 hours the ante-
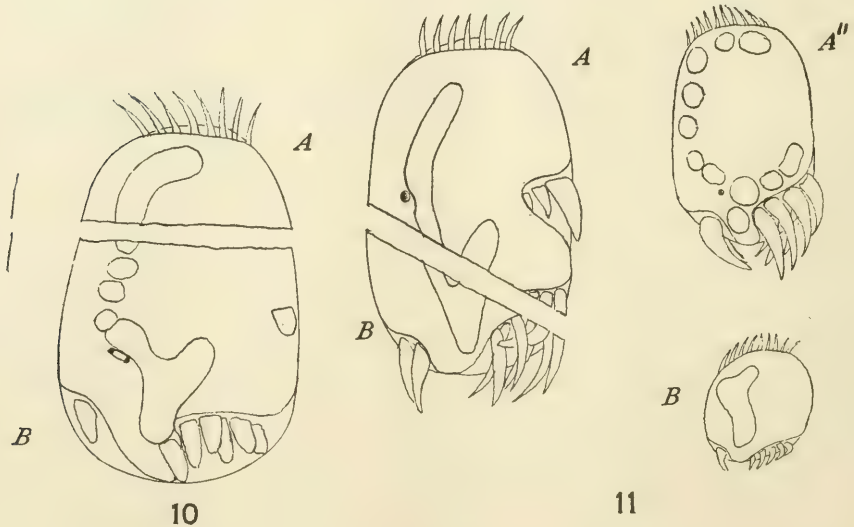
Fig. 10 Experiment 12. Cell at the onset of division. Fragment A regenerated in 18 hours. B divided in the original plane of division forming one minute cell from the anterior end, and a perfect cell from the posterior end.

Fig. 11 Experiment 75. Cell at the mid-phase of division. Fragment A divided in the original division plane into A' and A'', the latter developed into a minute but perfect cell in 24 hours, the former into a normal large cell. B formed a small but perfect cell without a micronucleus (B below on right). A'' represents the posterior fragment of A 42 hours after cutting.

rior part A had divided into a normal A' and a minute A'', while the posterior part B had regenerated into a perfect cell.   After 42 hours A' and A'' were nearly perfect and were killed for internal structures.   In experiment 25 three perfect cells were formed in a similar manner, but in no. 16 one fragment (A) died before 24 hours.

B. *In the mid-phase of division.* At this period the macronucleus is fully condensed and ready for division, while the micronucleus may or may not be divided.

In experiments 66 and 72, the cells were cut longitudinally. In no. 66 after 6 hours, A had started to regenerate while B had divided into B′ and B.″ All were then killed and B′ found to have a micronucleus; B″ was a mere fragment with disintegrated macronucleus; A had long membranelles and new cirri, but no micronucleus at all (see fig. 12). In no. 72 the cell was
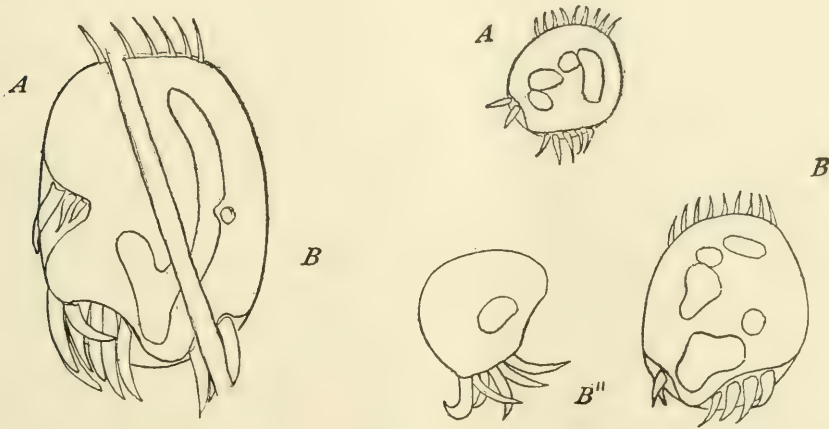
Fig. 12 Cell at the mid-phase of division cut longitudinally. A developed into a single individual with perfect motile apparatus but without a micronucleus (A on right). B divided, forming B′ and B″ (on right).

cut as shown in fig. 13. After 6 hours A was slowly regenerating, B had divided into B′ very small, and B″ normal. After 24 hours A and B″ were perfect but B′ had not regenerated. Lost by accident.

In experiments 2, 7, 22, 58, 63, 67, 68, 69 and 70, the cells were cut transversely. In nos. 22, 58 and 67, the plane of the cut coincided with the plane of division and in each of these cases only two cells were formed, each perfect. In Experiments 2, 7, 63, 68 and 69, the plane of the cut was either anterior or posterior to the plane of division and the larger fragment divided in the
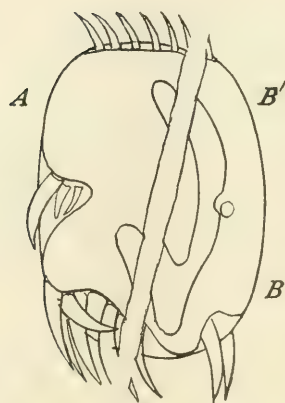
13
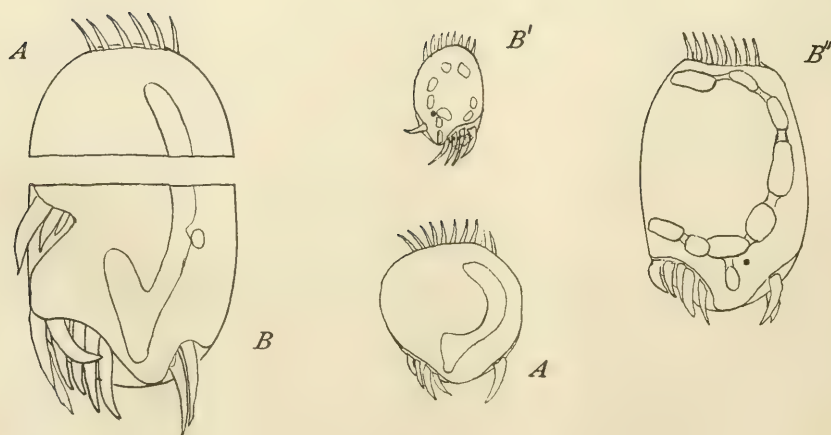
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Fig. 13   Experiment 72.   Cell cut at the mid-phase of division. B divided in the original plane into B and B'. A formed a single perfect cell but without micronucleus.

Fig. 14   Experiment 69.   Cell cut at the mid-phase of division. A, B' and B'' on right show the results after 24 hours, B having divided into B' and B''. A shows the typical nuclear features of a cell just after division, but has no micronucleus.

original plane in every case. Of this set, no. 69 is typical and well illustrates the reaction to the operation at this period of division. The cell was cut as shown in fig. 14, above the line of division. After 6 hours A was complete and B had divided, B' being very small and with only six cirri, while B'' was complete and normal. After 24 hours A was completely regenerated; B' one-eighth size and transparent but normal in structure; B'' was perfect. All were killed. A, B' and B'' had perfect macro-nuclei, but A had no micronucleus.

In experiment 70, alone, regeneration failed. The cell was cut just above the plane of division. After 6 hours A divided in the original plane, and B was perfect. After 24 hours neither A' nor A'' were regenerating and all were killed. The macronucleus in B was degenerating, A' had a perfect macronucleus and micronucleus and would have regenerated if not killed. A'' had no nucleus at all and only half of an adoral zone, but had large cirri.

In experiment 59 the cell was cut obliquely and in such a way that only a small fragment of the macronucleus was included. After 4 hours A had not regenerated while B had divided. After 24 hours A was perfect, so also was B', but B'' was only one-eighth size and not fully regenerated.

C. If cut during the later phases of division. In experiments 10, 35, 60 and 62 the cells were cut after division of the micronucleus and during division of the macronucleus. The cleavage furrows were conspicuous, indicating a condition of approximately half to three-quarters of an hour before final separation.

In nos. 10 and 35, the plane of the cut was close to the plane of division. In both cases only two cells were formed and one cell in each case became abnormal after 24 hours. In these cases the macronucleus was degenerated and distributed throughout the cell.

In experiment 62 the cell was cut longitudinally through the center as shown in fig. 15. After one-half hour the fragments were nearly divided; after one hour both were divided. After 24 hours all four fragments were very lively but none had regenerated. After 48 hours A' was dead while the rest were still unregenerated. All were then killed and no nucleus was found in any fragment although there was much chromatin in granule form distributed

about the cells. In experiment 60 the cell was cut obliquely through the anterior half. After 4 hours B had divided into a fragment $B,'$ and a normal cell $B.''$ After 24 hours A and B'' were large and perfect and B' was perfect but of only one-sixth size.

D. *If cut just prior to separation.* Experiments 26A, 26B, 27A, 27B, 50 and 65 were on cells almost ready to separate. The nuclear conditions, therefore, were similar to those of the cells described in section A. In no. 26 the dividing cell was first cut
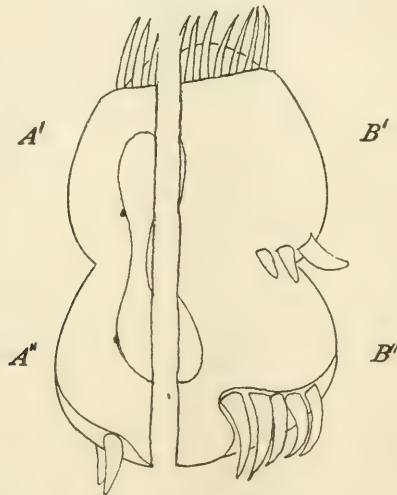
Fig. 15 Experiment 62. Cell cut during a late phase of division. Each fragment subsequently divided in the original plane of division, but none of the four parts regenerated in 48 hours.

through at the delicate point of attachment (cf. fig. 3A) Cell A was then cut longitudinally through the center. After 2 hours A' had three cirri and a nearly complete adoral zone A'' had five cirri and one-half the adoral zone. After 18 hours A' was complete and normal, A'' had the same 5 cirri and half the adoral zone and failed completely to regenerate. Cell B (26B) was also cut longitudinally through the center. After 2 hours B' had three cirri and three-fourths of an adoral zone; B'' had five cirri and three membranelles. After 18 hours B' was complete and normal but small; B'' has not regenerated, and did not regenerate later.

In experiment 27 the nearly divided cell was cut through the connecting strand as in no. 26. In 27A the second cut was longitudinal. After 18 hours A' remained a mere fragment while A'' had six cirri and an abnormal peristome. After 42 hours A' had not regenerated at all and A'' was imperfect in shape although complete. No. 27B was also cut longitudinally, B' being very minute was lost. After 18 hours B'' had seven cirri and part of the adoral zone. After 42 hours it had not regenerated further and was killed.

In experiment 50 two halves of a dividing cell were separated artificially as before. A was cut longitudinally, while the sister cell was lost. In 24 hours A' had regenerated into a one-quarter sized individual while A'' failed entirely to regenerate. In 72 hours A' had reached full size and was normal in all ways.

In experiment 65 the posterior part was cut obliquely through the macronucleus. After 24 hours B' was one-fourth, B'' was one-eighth size and both were normal.

E. Cells cut two or more times during division. In experiment 6 the cell was in the mid phase of division and cut twice, the central fragment being lost. The anterior fragment died within 24 hours, but the posterior fragment regenerated perfectly.

In experiment 17 the cell was in the early phase of division when cut twice. The anterior fragment was little more than the adoral zone and was lost; the central portion regenerated within 24 hours but the posterior fragment failed entirely to regenerate. After 72 hours the central portion divided. On killing and staining the posterior portion no nucleus was found.

Experiment 19 was more satisfactory and decisive. Here the dividing cell was first cut transversely through the adoral region of the anterior cell, and second, transversely just posterior to the original division plane. After one hour A had regenerated two big cirri, B' had normal but crowded cirri, and C had a new and complete adoral zone. After 18 hours A had abnormal cirri, B had no adoral zone, while C was normal and active. After 42 hours A had regenerated into a perfect cell of one-quarter size, B remained without regeneration but was lively, and C was normal. After 66 hours A was dead, B remained the same and after

90 hours B was dead without regenerating. This experiment is interesting because the central and largest piece not only failed to divide through the original plane of division, but also failed to regenerate.

Experiment 30 was in connection with a cell in the early phase of division. The first cut was through the posterior part of the macronucleus, the second was longitudinally through the anterior fragment. After 18 hours A' was complete and normal. A'' was not regenerated while B was complete but with a large cirrus arising from the middle of the adoral zone. After 42 hours both A' and A'' were complete and normal while B remained as before. In no. 40 a form in the mid phase of division, all three parts were alive but not regenerated after 24 hours. The next day two of the three were dead and the third not regenerated, and this too, died before the end of the 73d hour.

Similar results were obtained with other forms cut two or more times, the larger pieces of dividing forms regenerated, the smaller ones not, and in some cases no regeneration occurred.

GENERAL

These experiments indicate that in Uronychia the power of regeneration is greatest at the time of division. They indicate, furthermore, that something is present in the cell body at this period which is not present at other times. The organism, if cut immediately after division has a very limited power of regeneration, and then only when both parts of the nuclear apparatus are present. In some cases even when the cell is provided with all of the cellular organs, as in experiment 42 (fig. 4A, p. 101), regeneration failed; or again, as in no. 43 where a portion only was removed from the peristomial region, the cell failed completely to respond. Fig. 3B shows that the nucleus is still condensed immediately after division, after a couple of hours, however, the characteristic form of the resting nucleus is assumed and growth has begun, while after from six to twelve hours the cell is full-sized and normal. Even at this period however, the power to regenerate is very limited as shown in section 3. Here, out of ten cases in which both parts were retained, only two resulted in complete

regeneration of both parts, and in one of these (no. 37, the smaller one died in 48 hours, so that only one (no. 21) gave two cells that continued to live and in this case the organism was 24 hours old when cut and probably about ready to divide. In all of the other cases one part only regenerated normally. It is almost impossible to cut these normal vegetative cells in such a way that no macronucleus is retained, but the micronucleus, being single, is invariably absent in one of the fragments. The power to regenerate therefore, is only feebly developed during the vegetative period. Out of 24 cases cut after the division period, only two gave complete regeneration of both parts.

In striking contrast to this result are the results of cutting during the period of division, counting as the period of division the time from the first external evidence (precocious formation of the new cirri) until final separation. In all there were 37 experiments on cells at this time, 7 of which were in the early stages, 14 in the mid stages, 8 in the last stages, while 8 were multiple cuts.

Analyzing these separately we find that of the seven cases cut immediately before division, six continued the division in the original plane forming two perfect cells in each case while the third part, or portion cut off, formed a perfect cell in five cases. In the majority of cases therefore, the original cell was made to form three perfect cells so far as general form and motile apparatus are concerned, and one of these parts in each case was without a micronucleus and must have regenerated with only a portion of the macronucleus.

Of the fourteen cases cut during the mid phase of division, only six formed three perfect cells each. Of the others, sometimes one, sometimes the other fragment failed to regenerate, although macronucleus material was present (in three cases the cut was directly through the plane of division so that three cells were not formed; in each of these two perfect organisms resulted.)

No general rule was observed in regard to the regeneration of cells cut during the late phases of division. When regeneration occurred, vitality of the regenerated parts seemed low and the life of the organism was short. Thus in experiment 10 both cells were perfect in 4 hours, but one portion (B) soon became abnormal and when killed after 72 hours showed a degenerated macronu-

cleus. A similar result was obtained in no. 35. In no. 62 the dividing cell was cut longitudinally; division was continued and each fragment divided in the original plane thus forming four fragments, not one of which regenerated.

Of the four cases cut at the end period of division, only one formed three perfect cells. All of the others behaved like cells cut immediately after division.

Of the eight cases of dividing forms in which the cell was cut twice the results conformed with those of the dividing period generally, although smaller pieces failed to regenerate and even larger pieces including the original plane of division and much macronuclear material failed to regenerate (see no. 19).

Further evidence of the limitation of the maximum regenerative power is given by the comparison of cells cut in exactly the same relative portions of the cell, but at different periods of vegetative life. Double regeneration occurred only when the cells were at or near the beginning of the division period, never after division.

One general result obtained from these experiments is that Uronychia when cut does not divide as soon as the cell would have divided had it not been cut. The average division rate of these organisms under normal conditions is two divisions in three days; in the cut forms the usual period required to divide is never less than three days, or more than twice as long as the normal. Only a few controlled experiments were undertaken with this point in view and these are tabulated below:

EXPERIMENT NO.	AGE SINCE LAST DIVISION	TIME OF REGENERATION	TIME BEFORE DIVISION	TIME OF DIVISION OF CONTROL
		hours		*hours*
15	18 hours	6–12	No division in 72 hours	48
21	6–12 hours	8–12	72 hours	36
34	12–18 hours	10–20	None in 72 hours	36
39	2 minutes	12–18	None in 72 hours	36–42
46	15 minutes	12–20	None in 144 hours	48
51	1 hour	15–24	None in 72 hours	36
57	2 minutes	12–18	None in 36 hours	24
72	division	12–20	Divided in 72 hours	24–36

If cell division were merely a matter of mass relation of cell body to nucleus then by cutting the cell with only a small portion of the nucleus this relation would be changed to the advantage of the nucleus. We should expect, on Hertwig's theory that the regulatory process of the cell which brings about cell division would be operative under these changed conditions, but such is not the case. Division instead of being hastened, is retarded and in some cases, prevented altogether.

Wallengren's careful and accurate description of the regenerative processes in hypotrichous ciliates, including Uronychia, shows that all of the appendages are discarded and new ones formed in the cell before division occurs. This regeneration which for Uronychia, I have amply confirmed, occurs before the nucleus is entirely concentrated, (cf. fig. 2) and before the nucleus divides. This indicates a condition in the cytoplasm at this time not present at other times, a condition which may be due to the presence of substances at certain periods of cell life. This condition at times of division is analogous to the condition at times of regeneration, that is, regeneration is equivalent to this precocious cirri and membranelle formation at division. We have seen that the power to regenerate is much reduced at periods immediately after division and then only when the full complement of micro- and macronucleus is present; we have seen furthermore, that this power increases towards the next division period and is at its maximum at the time of division.

These facts are best interpreted on the supposition that substances are formed in the nucleus and transferred to the cytoplasm where they or the products of their activity, accumulate until a condition analogous to saturation is reached. Cutting the cell at this period results in the exhaustion through regeneration, of these substances so that cell division is retarded in forms where the division process is not already started. The facts, therefore, lend support to deVries' and Hertwig's view that the nucleus gives off to the cytoplasm during the vegetative stages, certain formative substances perhaps of the nature of enzymes, which are exhausted with the regenerative processes accompanying division.

During vegetative life the fragmentation of the macronucleus into chromatin spheres gives a much increased surface for the interaction of nucleus and cytoplasm. At the time of division these spheres unite into a single compact nucleus with considerable loss of material, analogous perhaps, with that formed previously and this material, in the form, possibly, of nucleoproteids, may be the activating substance which underlies the regenerative power of the cell.

Columbia University
    December, 1910

STUDIES IN THE LIFE CYCLE OF HYDATINA SENTA

II. THE RÔLE OF TEMPERATURE OF THE CHEMICAL COMPOSITION OF THE MEDIUM, AND OF INTERNAL FACTORS UPON THE RATIO OF PARTHENOGENETIC TO SEXUAL FORMS[1]

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INTRODUCTION

In two earlier papers (Shull, '10a and '10b) I have discussed the influence of environment upon the transition from the parthenogenetic to the sexual mode of reproduction in Hydatina[2] senta.

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[1] I desire to thank Prof. E. B. Wilson for the use of laboratory facilities at Cold Spring Harbor, Long Island, where much of the work described in this and my former paper was done.

[2] It has been pointed out to me by several zoölogists independently that the name Hydatina can not stand as a genus of rotifers, that name having been previously used for a mollusk.

The life cycle of this rotifer and the substance of the previous literature on the subject were given in the second paper. Experiments were there described from which the conclusion was drawn that the mode of reproduction was not directly influenced by starvation, nor probably by temperature differences; but that the chemical content of the water in which the rotifers lived had a strong influence upon the proportion of sexual females (male-producers).

In the present paper the nature of the substances involved is more definitely stated. I shall name a few single substances which have been found to influence the mode of reproduction, and give the evidence for my conclusion. Among these substances are several ammonium compounds, creatin, probably urea, and perhaps the degree of alkalinity. Certain internal factors have likewise been found to influence the proportion of sexual females, and these have been variously combined in crosses.

This work has been continued with helpful suggestions from Prof. T. H. Morgan. I am also indebted to Prof. William J. Gies for suggestions regarding the chemical part of the work.

PROBLEM AND METHOD

In my earlier paper (Shull, '10b) no evidence relating to the influence of temperature differences on the proportion of sexual females was given, because further experiments were in progress at that time. These experiments are now completed. The principal problem, however, was to discover the effect of various substances in the water upon the proportion of sexual females; for it had been found that if the rotifers were bred in a fairly concentrated solution of horse manure, sexual females might be wholly prevented from appearing. Such a manure solution contains a large number of substances, but it was not known which of these substances affected the life cycle.

It also seemed important to search for internal factors which might likewise influence the proportion of sexual females, for some students of this and other similar life cycles believe that internal factors play a leading rôle in the production of the cycle. Punnett

TABLE 17

Showing number of male-producers and female-producers in the progeny of two sister individuals of Hydatina senta, the one line being reared at a temperature of 18° to 23° C., the other at 23° to 26° C. Male-producers are designated ♂♀, female-producers ♀♀.

	18° TO 23° C.				23° TO 26° C.		
NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀
1	Oct. 8	37	2	1	Oct. 7	0	13
2	10	11	34	2	9	1	6
3	12	13	32		10	3	9
4	14	2	12	3	11	6	20
5	15	0	31	4	12	3	28
6	17	0	35	5	14	19	13
7	18	0	48	6	15	1	15
8	20	10	4	7	17	1	6
9	21	0	17	8	18	4	14
10	23	4	27	9	20	18	12
11	25	9	34		20	2	31
12	27	2	17	10	21	17	11
13	28	31	13	11	22	7	16
14	30	6	32	12	24	4	18
15	31	0	14	13	26	7	18
	Nov. 1	8	20	14	27	14	10
16	2	1	23	15	30	3	30
17	3	0	43	16	31	4	15
18	5	4	33	17	Nov. 2	0	3
19	8	12	14		3	0	12
20	10	2	5	18	4	2	4
	12	2	9		4	5	5
21	12	2	11	19	5	0	7
	13	0	19	20	7	2	24
22	15	0	32	21	8	0	21
23	18	1	31	22	10	5	25
	18	2	0	23	11	4	12
				24	13	0	17
				25	14	1	10
				26	16	1	11
					16	2	35
				27	18	1	29
				28	19	6	26
Total		159	592			143	526
Percentage of ♂♀		21.1				21.3	

('06) was inclined, in fact, to believe that external agents have no influence on the cycle of Hydatina, and that many of the phenomena observed by him were attributable to internal differences in distinct pure lines. It was shown in my earlier paper, cited above, that many phenomena are not due to internal differences; but it was left an open question whether internal differences might not explain other results. If distinct pure lines could be found, it was proposed to test the nature of their internal differences by appropriate crosses.

The method of conducting the experiments has been in all essential respects the same as that described in the previous article.

EXPERIMENTS WITH EXTERNAL FACTORS

Influence of temperature on the percentage of male-producers

Experiment XV. On October 6, 1909, a female was taken from the pure line recorded in Experiment I of my former paper, which was being reared at room temperature (18° to 23° C.), and was placed in a dish in a closed box kept on or near a large water bath. The temperature in this box was noted frequently, and found to vary between 23° and 26° C. A higher temperature could not be maintained without such great mortality that the experiment would have small value. This pure line, and the control derived from a sister individual and reared at 18° to 23° C., are recorded in table 17. The difference of temperature here used, which averaged 4° or 5° C., had practically no effect upon the proportion of male-producers.

Experiment XVI. Simultaneously with the beginning of the preceding experiment, a line of rotifers was removed to an ice-chest, where the temperature was found to vary between 7° and 14° C., nearly always between 9° and 11° C. The same control was used as in the preceding experiment, the three lines being derived from sisters. Table 18 gives the data.

The line bred at the lower temperature yielded a very much higher proportion of male-producers. This result is opposed to that of Maupas ('91), who found that the higher temperature resulted in the greater proportion of male-producers. Because of

TABLE 18

Showing number of male- and female-producers in the progeny of two sister individuals of Hydatina senta, one line being reared at a temperature of 18° to 23° C., the other at 7° to 14° C.

	18° TO 23° C.				7° TO 14° C		
NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀
1............	Oct. 8	37	2	1	Oct. 8	22	11
2............	10	11	34	2	12	10	32
3............	12	13	32	3	16	15	17
4............	14	2	12	4	20	24	22
5............	15	0	31	5	25	5	30
6............	17	0	35	6	30	11	24
7............	18	0	48		30	14	19
8............	20	10	4	7	Nov. 6	13	2
9............	21	0	17		6	9	27
10............	23	4	27		6	16	28
11............	25	9	34		6	5	19
12............	27	2	17	8	12	7	26
13............	28	31	13		12	9	21
14............	30	6	32	9	16	7	20
15............	31	0	14		16	19	7
	Nov. 1	8	20	10	19	25	17
16............	2	1	23	11	24	16	20
17............	3	0	43		24	27	16
18............	5	4	33	12	30	30	15
19............	8	12	14		30	20	23
20............	10	2	5	13	Dec. 4	6	29
	12	2	9		4	33	1
21............	12	2	11	14	9	7	4*
	13	0	19		9	14	3*
22............	15	0	32				
23............	18	1	31				
	18	2	0				
24............	21	5	13				
25............	23	1	20				
26............	26	1	28				
27............	29	2	23				
28............	Dec. 3	0	37				
29............	5	0	30				
30............	7	1	20				
31............	10	1	21				
32............	13	0	3*				
Total..............		170	787			364	433
Per cent of ♂♀		17.7				45.6	

*Remainder of family not recorded.

this contradiction, it was deemed necessary, notwithstanding the very large difference obtained, to repeat the experiment.

Experiment XVII. The temperature experiment was twice repeated, on a less extensive scale, during the time when the principal experiment was in progress. In one repetition (A, table 19) a female was removed from room temperature to the ice-chest; in the other (B), the female was removed from the ice-chest to room temperature. Sister individuals became the parents of the two lines in each case.

TABLE 19

Showing data of two temperature experiments, in each of which one line was bred at 18° to 23° C., the other at 7° to 14° C.

EXPERIMENT	18° TO 23°C.				7° TO 14°C.			
	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
A	1	1	20		1	1	27	
	2	1	28			1	26	
	3	2	23		2	0	8	
	4	0	37			0	15	
	5	0	30		3	2	21*	
	6	1	20			7	26*	
	7	1	21		4	9	3*	
	8	0	3*					
Total		6	182	3.1		20	126	13.6
B	1	9	36		1	16	20	
	2	14	28			27	16	
	3	19	22		2	30	15	
	4	7	35			20	23	
	5	2	44		3	6	29	
	6	1	28			33	1	
	7	9	26		4	7	4*	
	8	5	17			14	3*	
	9	6	6					
		5	28*					
	10	16	14*					
Total		93	284	24.6		153	111	57.9

*Remainder of family not recorded.

The results of both repetitions agree with that of the preceding experiment, in yielding more male-producers at the lower temperature. It seemed important to test whether the same would occur with other pure lines and at different times. The next experiment was performed after all the preceding ones were concluded, and with rotifers that were probably not in any way related to those previously used.

Experiment XVIII. From an old stock jar in the laboratory, which had been stocked with Hydatina about two years and a half before the opening of this experiment and to which none had been added since, a rotifer was isolated in January, 1910. Of its daughters, two became the parents of the two lines in table 20, one of which was reared at room temperature (20° to 22° C.), the other in an ice-chest at 7° to 11.5° C.

Here the difference is in the opposite direction; the higher proportion of male-producers is produced at the higher temperature. The meaning of these seemingly opposite results is discussed elsewhere. In connection with that discussion, it is important to note the sudden rise in the proportion of male-producers at room temperature at the end of January, and the corresponding smaller rise at the lower temperature early in February.

Influence of various undetermined constituents of feces on the percentage of male-producers

After it had been determined that a solution of horse manure could wholly prevent the appearance of male-producers, the immediate problem was to discover what constituent or constituents of the feces had this effect. Two methods of investigation were practicable. Substances which were known to be present in horse manure, or in feces in general, could be directly tested by experiment; or the solution of manure could be treated in such a way as to remove from it substances having given properties, after which the effect of the substances removed, or of those that remained, or of both, could be determined. By the latter method, the number of substances to be tried by the former method might be considerably reduced. It is this indirect method which is the subject of

　　　　　　A. FRANKLIN SHULL

TABLE 20

Showing number of male- and female -producers in the progeny of two sister indi-
viduals of Hydatina senta, one line being reared at 20° to 22° C., the other at 7°
to 11°.5 C.

| | 20° TO 22° C. | | | | | 7° TO 11°.5 C. | | | |
NO. OF GENERA- TION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF GENERA- TION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
1..........	Jan. 15	0	43		1	Jan. 15	0	25	
2..........	17	0	47		2	20	1	49	
3..........	18	3	22		3	24	0	51·	
4..........	20	0	49		4	27	0	52	
5..........	21	0	1			27	1	49	
	21	0	36		5	31	0	52	
6..........	23	0	20			31	1	48	
7..........	25	1	44		6	Feb. 4	4	39	
8..........	26	0	45			4	7	38	
9..........	27	7	40		7	7	1	40	
10..........	29	0	21		8	12	0	49	
11..........	31	24	25		9	17	4	45	
12..........	Feb. 2	10	37		10	22	5	44	
13..........	4	0	44			22	3	43	
14..........	5	0	36		11	26	0	43	
15..........	7	0	2		12	Mar. 3	0	50	
16..........	9	0	44		13	7	1	38	
17..........	11	0	3						
18..........	13	0	34						
19..........	15	5	32						
20..........	16	7	24						
21..........	18	1	39						
22..........	19	0	3						
	21	3	35						
23..........	21	1	7						
	22	0	2						
24..........	22	0	7						
	23	0	17						
25..........	24	1	47						
26..........	26	11	42						
27..........	27	11	26						
28..........	Mar. 1	9	43						
29..........	2	6	39						
30..........	4	6	37						
31..........	5	4	44						
32..........	7	1	43						
Total........		111	1080	9.3			28	755	3.5

the present section.   The manure solution was boiled; it was evapo-
rated to dryness and redissolved; it was evaporated to dryness and
extracted with ether and alcohol; and it was decolorized by boil-
ing with animal charcoal.   The following experiments show the
results of these operations in detail.

Experiment XIX.   Boiling the manure solution.   After the
manure solution from an old food culture, made up with spring
water, had been filtered through a Berkefeld filter, part of the
filtrate was boiled gently for four minutes.   The loss of volume by
evaporation was restored with distilled water.   The remainder of

TABLE 21

*Showing the number of male- and female-producers in the progeny of three sister
individuals of Hydatina senta, one line being bred in boiled filtrate of old food
cultures, one in unboiled filtrate, the third in spring water.*

SPRING WATER			UNBOILED FILTRATE			BOILED FILTRATE		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
1	27		0	7		0	42	
23	27		0	2		0	51	
5	46		0	4		0	47	
7	42		0	2		0	47	
1	34		0	57		0	32	
			0	26				
			0	42				
37	176	17.3	0	140	0.0	0	219	0.0

the filtrate was not treated.   The boiled filtrate was kept in stock
and was boiled every day until exhausted, chiefly to prevent the
development of bacteria.   The unboiled filtrate was secured daily
just before using.   Three pure lines of rotifers derived from sisters
were reared in April, 1910, one in boiled filtrate, one in unboiled
filtrate, the third in spring water.   The results are given in table
21.   The boiled filtrate has precisely the same effect as the un-
boiled.   The substance in feces which prevents male-producers
from appearing is not, therefore, an enzyme, nor bacteria, nor
any other substance destroyed by boiling temperature.

Experiment XX. Drying the manure solution. A portion of the filtrate obtained from an old food culture, made of spring water as in the preceding experiment, was evaporated to dryness. The residue was redissolved in distilled water equal in volume to the original filtrate, and then boiled. One line of rotifers, bred from a female collected at Grantwood, N. J., in April, 1910, was reared in this redissolved filtrate. A second line, from a sister to the parent of the other line, was reared in the filtrate that had been simply boiled. Another line was bred in spring water. The data of the three lines are given in table 22. The substance responsible for repressing the male-producers is not destroyed by drying and re-

TABLE 22

Showing number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being reared in the filtrate of old food cultures that had been dried and redissolved, one in boiled filtrate, and one in spring water.

SPRING WATER			BOILED FILTRATE			DRIED, REDISSOLVED FILTRATE		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
0	16		0	6		0	3	
3	11		0	15		0	42	
0	9		0	13		0	39	
15	20		0	12		0	43	
1	18		0	16				
			0	1				
			0	31				
19	74	20.4	0	94	0.0	0	127	0.0

dissolving, for the redissolved filtrate has precisely the same effect as the merely boiled filtrate.

Experiment XXI. Ether- and alcohol-soluble parts of manure solution. From an old food culture, made as in the preceding experiments from spring water, 125 cc. was filtered through a Berkefeld filter, and the filtrate evaporated to dryness. The residue was extracted with ether for twelve hours, after which the ether was filtered through paper and the solution evaporated to dryness. Less than 0.01 gram of ether-soluble substances was thus obtained. It did not seem likely that so small a quantity

would have any noticeable effect on the proportion of male-producers, but the experiment was nevertheless made. This ether-soluble residue was dissolved in 125 cc. of distilled water, giving a clear solution, and a line of rotifers was reared through four generations in it. The residue after ether-extraction was likewise dissolved in 125 cc. of distilled water and boiled, making a brown solution not apparently different from the original manure culture, and a sister line of rotifers was reared in the solution. A third line was reared as control in spring water. Table 23 shows the result.

TABLE 23

Showing the number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being bred in a solution of the ether-soluble part of an old food culture, one line in the part insoluble in ether, the third in spring water.

SPRING WATER			ETHER SOLUBLE			NOT ETHER SOLUBLE		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
0	16		0	29		0	4	
3	11		4	16		0	24	
0	9		5	44		0	20	
15	20		1	12		0	14	
1	18					0	43	
19	74	20.4	10	101	9.0	0	105	0.0

The experiment was so brief that the difference in the proportion of male-producers between the line in spring water and that in the ether-soluble part of the filtrate may mean nothing. The chance of obtaining any result from such a minute ether-soluble residue did not seem to warrant a more extensive experiment, especially since the part of the manure solution not soluble in ether had the same effect as the entire manure solution had in other experiments.

An experiment was started, in which the alcohol-soluble portion of the manure solution was obtained in a manner similar to the ether-extraction above. The portion soluble in absolute alcohol was smaller than that soluble in ether. The experiment was dis-

continued in the middle of the second generation, hence the detailed results are not of value. It may be said, however, that the first family reared in the alcohol-soluble portion contained a considerable number of male-producers.

Experiment XXII. Decolorized filtrate. It was noticed in the preceding experiments of this section that those residues which produced the same effect as the unaltered manure solution were brown like the original; those that had no effect were colorless. To determine whether the substance producing the brown color has any effect on the life cycle of the rotifers, a portion of the filtrate from old food culture was decolorized with animal charcoal. An excess of the charcoal was added to the filtrate, and the mixture boiled eight or ten minutes. It was then filtered through paper, and the volume of water lost by evaporation was restored, the added water being passed through the filter. More charcoal was added to the filtrate, and the boiling repeated. After three or four boilings the filtrate was practically colorless. It was not to be expected that the colored substance was the only one removed by this process for animal charcoal probably carries down most substances to some extent. But if the colored substance is wholly responsible for the non-occurrence of male-producers in a manure solution, such a decolorized solution should yield the same proportion of male-producers as pure water. Whether it does or not may be seen from table 24. The experiment was performed in May, 1910, with rotifers descended from a winter egg collected in Grantwood, N. J., in April, and kept in an ice-chest for a month.

Even the decolorized filtrate greatly reduces the proportion of male-producers, though not as much as the merely boiled filtrate. Whether the 7 per cent difference between the decolorized filtrate and the boiled filtrate is due to total removal of the colored substance or to partial removal of some other substances, can not be decided from this experiment alone. That the latter is the case, at least in part, will appear later, when it is shown that certain other substances which are presumably present in the manure, and which were probably carried down mechanically by the charcoal, do reduce the percentage of male-producers. This does not

show, however, that the colored substance may not also have the same effect to a slight extent.

The one male-producer in the first generation bred in the boiled filtrate is the only one I have ever obtained in undiluted filtrate of old food cultures. This one individual need scarcely surprise us if we note the very high percentage of male-producers in the first two generations bred in spring water. Manure solution does not rigidly exclude male-producers. It is important to

TABLE 24

Showing the number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being reared in boiled filtrate of old food cultures, one in filtrate decolorized with animal charcoal, the third in spring water.

SPRING WATER			DECOLORIZED FILTRATE			BOILED FILTRATE		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
31	6		3	39		1	42	
20	5		3	29		0	9	
7	21		0	6		0	14	
6	23		8	27		0	22	
6	39		2	37		0	14	
16	14		1	30		0	38	
30	10		4	45		0	13	
0	41		0	48		0	20	
						0	5	
116	159	42.1	21	261	7.4	1	177	0.5

remember this in the consideration of other experiments where various substances do not wholly prevent male-producers from appearing.

Influence of alkalinity on the percentage of male-producers

Experiment XXIII. It was found impracticable to rear the rotifers in water that was even faintly acid, so several degrees of alkalinity were used. A $\frac{N}{10}$ solution of NaOH in distilled water was kept in stock in a tightly stoppered bottle. In making up this solution, no further precaution was taken to make the normality exact than to weigh the solid hydroxid carefully, exposed

to the air.   A $\frac{N}{25}$ solution of HCl was made up by titration against the NaOH solution immediately after the latter was prepared, and was kept in stock.   Three grades of alkalinity were used in the experiment, the solutions being prepared as follows: Great Bear spring water, which is itself alkaline, was used unaltered in one line; for the second line, the $\frac{N}{10}$ NaOH solution was diluted to ten times its volume with Great Bear water; for the third line, the $\frac{N}{25}$ HCl solution was diluted to forty times its volume with Great Bear water.   The alkalinity of the Great Bear water was not

TABLE 25

Showing number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being bred in Great Bear spring water, one in water less alkaline, and the third in water more alkaline, than Great Bear water.

LOWER ALKALINITY			GREAT BEAR WATER			HIGHER ALKALINITY		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
0	31		7	24		1	36	
0	27		0	21		3	31	
14	16		0	18		0	39	
0	16		0	18		0	3	
5	18		2	22		1	5	
0	26		11	4		11	15	
0	3		0	32		0	24	
0	10		0	40		0	10	
0	27		7	40		1	21	
6	27		1	25		0	2	
1	9							
26	210	11.0	28	244	10.2	17	186	8.3

accurately determined, but was less than $\frac{N}{10}$, so that addition of $\frac{N}{10}$ NaOH increased its alkalinity; neither was the alkalinity of the diluted solutions known.   As it was necessary to expose the NaOH solution to the air while it was being used, the dilute solution was made up daily.   The data from this experiment are given in table 25, where the alkalinity increases from left to right.

From this experiment it would seem that the greater the alkalinity the fewer the male-producers, but the differences are too small to draw a safe conclusion from one experiment.

Experiment XXIV. The stock solutions in this experiment were produced in essentially the same manner as in the preceding experiment, but in diluting them for daily use distilled water was used instead of Great Bear water. The alkalinity of the final solutions was therefore more accurately known. It could not be exactly known, however, for the food cultures were of varying alkalinity, and, because the protozoa in them were not always equally abundant, a variable quantity of the cultures was necessarily used.

TABLE 26

Showing number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being reared in $\dfrac{N}{200}$ NaOH, one in distilled water, and one in $\dfrac{N}{300}$ HCl (but see text).

$\dfrac{N}{300}$ HCL			DISTILLED WATER			$\dfrac{N}{200}$ NaOH		
NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
1	35		0	47		3	28	
0	23		1	32		3	52	
1	10		5	47		6	46	
13	38		3	42		4	48	
5	20		1	22		0	48	
0	15		0	55		0	49	
1	21		4	34		1	25	
0	32		0	48		4	34	
			5	40				
21	194	9.7	19	367	4.9	21	330	5.9

Before adding food to the dishes, the three solutions used were respectively $\frac{N}{200}$ NaOH, neutral (distilled water), and $\frac{N}{300}$ HCl. After adding food, the acid solution was practically neutral or faintly alkaline, while the alkalinity of the other two solutions was slightly altered. In table 26 the columns are designated according to the acidity or alkalinity of the solutions before adding food, but it should be remembered that an actually acid solution was never used.

While the highest percentage of male-producers is found as before in the lowest degree of alkalinity, there is not the regular decrease in the proportion of male-producers with increase of alkalinity, such as was seen in the preceding experiment. As it would not be practicable to rear the rotifers in much more alkaline water, all that we may safely conclude is that if alkalinity influences the proportion of male-producers it does so only to a small extent.

Influence of urea on the percentage of male-producers

Experiment XXV. One line of rotifers was bred in a $\frac{M}{400}$ solution of urea, a control line derived from a sister individual was reared in spring water. The experiment was performed twice, A and B, in table 27. In A, the parents of both lines had been in spring water before the beginning of the experiment; in B, both had been in urea. This experiment seems to indicate that urea in the water tends to reduce the proportion of male-producers.

Influence of ammonium compounds on the percentage of male-producers

Experiment XXVI. Ammonium salts. Four sister individuals became the parents of the four lines in this experiment, which was performed in June, 1910. One line was reared in spring water, the others in ammonium salts of the following strengths: $\frac{M}{300}$ NH_4Cl, $\frac{M}{300}$ NH_4NO_3, and ammonium carbonate 1 gram to 7500 cc. of the solution. The carbonate was not computed in terms of molecular solution, because the c.p. salt was not used. The substance used was probably what is known as the sesquicarbonate, a mixture of the bicarbonate and the carbamate. Table 28 gives the details of each line.

All of these ammonium salts reduced the proportion of male-producers, two of them to one-half the proportion obtained in spring water, the other to one-third. The consistent results from the three salts seemed to make a repetition of the experiment with any one of them unnecessary.

TABLE 27

*Showing number of male- and female-producers in the progeny of two sister indi-
viduals of Hydatina senta, one line being bred in spring water, the other in a solu-
tion of urea. A and B are separate experiments.*

EXPERIMENT	SPRING WATER				$\frac{M}{400}$ UREA			
	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
A	1	0	2		1	0	4	
	2	0	44		2	1	28	
	3	0	3		3	0	5	
	4	0	34			0	4	
	5	5	32			0	10	
	6	7	24		4	2	34	
	7	1	39		5	0	40	
	8	0	3		6	0	33	
		3	35		7	1	46	
	9	1	7		8	0	4	
		0	2			0	2	
	10	0	7			7	24	
		0	17		9	0	1	
	11	1	47			0	1	
	12	11	42		10	0	12	
	13	11	26		11	1	16	
	14	9	43		12	5	51	
	15	6	39		13	1	33	
	16	6	37		14	2	42	
	17	4	44		15	0	1	
	18	1	43			0	2	
						0	18	
					16	0	27	
					17	0	35	
Total		66	570	10.3		20	473	4.0
B	1	5	11		1	5	51	
	2	1	49		2	1	33	
	3	17	35		3	2	42	
	4	0	23		4	0	1	
	5	5	25			0	2	
	6	5	6			0	18	
					5	0	27	
					6	0	35	
Total		33	149	18.1		8	209	3.6

TABLE 28

Showing number of male- and female-producers in the progeny of four sister individuals of Hydatina senta, one line being bred in spring water, the other three respectively in solutions of ammonium chloride, ammonium carbonate, and ammonium nitrate.

SPRING WATER		AMMONIUM SALTS					
		CHLORID		CARBONATE		NITRATE	
NO. OF ♂♀	NO. OF ♀♀	NO. OF ♂♀	NO. OF ♀♀	NO. OF ♂♀	NO. OF ♀♀	NO. OF ♂♀	NO. OF ♀♀
30	19*	1	15	4	23	10	28*
2	17*	8	26	7	8*	11	1*
0	2*	7	4*	2	11		
8	3	3	25	1	0	4	20
6	22	11	14		0	6	25
22	29	3	29	22	6	2	39
35	19	0	38	0	6	8	25
22	12	1	8	0	13	8	35
11	17	1	38	10	17	1	13
25	19	3	34	2	4	3	42
19	26	8	44	4	35	11	22
20	35	0	33	0	35	0	7
10	25	0	18	0	35	12	15
15	21	1	18	6	32	0	16
7	16	13	16	0	19		
				12	31		
				10	15		
232	296	60	360	80	290	76	288

Per cent of
♂♀ 43.9   14.2   21.6   20.8

*Remainder of family not recorded.

Experiment XXVII. Ammonium hydroxid. On July 6, 1910, a female from the same pure line as those of the preceding experiment was placed in a solution of ammonium hydroxid, made by diluting strong NH_4OH to 5000 times its volume with Great Bear spring water. Its progeny for thirteen generations were bred in the same solution. The strength of the original hydroxid was not known. Table 29 shows this line, and a control in Great Bear spring water.

The ammonium hydroxid, like the salts, reduced the proportion of male-producers to less than half that of the line in spring water. Here again it seemed unnecessary to repeat the experiment.

Influence of beef extract on the percentage of male-producers

The experiments with beef extract were designed chiefly to test the question whether extractives, which are present in feces, influence the proportion of male-producers. Liebig's extract was used, because of the absence from it of certain classes of substances which are present in other brands.

TABLE 29

Showing number of male- and female-producers in the progeny of two sister individuals of Hydatina senta, one line being bred in dilute ammonium hydroxid, the other in spring water.

SPRING WATER				AMMONIUM HYDROXID			
NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
1............	4	27		1	0	34	
2............	8	23		2	0	25	
3............	0	29		3	0	7	
4............	0	12		4	0	27	
5............	2	11		5	7	28	
6............	4	28		6	12	14	
7............	14	21		7	0	24	
8............	15	21		8	4	22	
9............	14	25		9	1	20	
	2	30		10	1	18	
	9	31		11	0	8	
10............	3	40		12	6	34	
	4	15			0	15	
11............	16	19		13	3	50	
	0	24					
12............	4	24					
13............	0	18					
14............	22	11					
Total........	121	409	22.8		34	326	9.4

TABLE 30

*Showing number of male- and female-producers in the progeny of two sister indi-
viduals of Hydatina senta, one line being bred in spring water, the other in a solu-
tion of beef extract.   A and B are separate experiments.*

EXPERIMENT	SPRING WATER				BEEF EXTRACT			
	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	PER CENT OF ♂♀
A	1	7	16		1	2	25	
	2	14	17			1	19	
	3	17	41		2	3	22	
	4	8	45			5	39	
	5	1	45		3	0	31	
	6	31	29			1	27	
	7	9	13		4	1	33	
	8	8	31			0	5	
	9	17	30		5	1	29	
	10	4	27			0	31	
	11	8	23		6	0	20	
	12	0	29			0	14	
	13	0	12		7	2	40	
						0	11	
					8	3	36	
						1	22	
					9	6	36	
						0	54	
					10	0	23	
						0	18	
					11	0	20	
						0	25	
					12	1	31	
						0	19	
Total		124	358	25.7		27	630	4.1
B	1	0	37		1	6	36	
	2	10	19			0	54	
	3	1	17		2	0	23	
		0	20			0	18	
	4	0	19		3	0	20	
		7	27			0	25	
					4	1	31	
						0	19	
Total		18	139	11.4		7	226	3.0

Experiment XXVIII.   Two sister females were isolated June 23, 1910, from one of the other experiments being performed at that time. One was placed in a solution of Liebig's beef extract, the other in spring water.   The beef extract was dissolved in spring water, and a 1 per cent solution kept in stock.   This stock solution was boiled once or twice a day to prevent the growth of bacteria, and in the intervals was set in the cool water of a spring. Loss by evaporation was restored with distilled water.   The stock solution was diluted with Great Bear spring water when ready to be used. In the first experiment, A, table 30, a 0.03 per cent solution was used for the first two generations, a 0.04 per cent solution thereafter.   In B, a female was removed from the line in beef extract in A to spring water, so that here also the strength of the beef extract is 0.04 per cent, the line in the extract being the last part of the corresponding line in A.

In both experiments there are considerably fewer male-producers in the beef extract than in spring water.

Experiment XXIX.   The preceding experiment was repeated, but this time two strengths of the extract were used, one a 0.04 per cent, the other a 0.05 per cent solution.   Both lines are represented in table 31.   The results of these repetitions agree closely with the first experiment, even showing a smaller percentage of male-producers in the stronger solution of the extract.   There can scarcely be any doubt that beef extract tends to reduce the proportion of male-producers, and it seems quite possible that these could even be wholly excluded by sufficiently strong solutions of the extract.

Influence of creatin on the percentage of male-producers

The positive results from the beef extract in the experiments of the preceding section suggested, among other things, that extractives may alter the proportion of male-producers.   One of the commoner extractives, creatin, was selected to put this matter to the test.   The creatin used in these experiments was not pure, but contained an admixture of creatinin.

TABLE 31

Showing number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being bred in spring water, the other two in solutions of beef extract of different strengths.

SPRING WATER			0.04 PER CENT BEEF EXTRACT			0.05 PER CENT BEEF EXTRACT		
NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀
1...........	8	23	1	0	21	1	0	20
2...........	0	29	2	0	23	2	0	13
3...........	0	12	3	0	15	3	0	9
4...........	2	11		0	17		0	4
5..........	4	28	4	0	13	4	0	11
6..........	14	21		0	15		1	12
7...........	15	21	5	0	26	5	0	18
8..........	14	25	6	0	19		0	19
	2	30	7	1	34	6	0	34
	9	31	8	4	34		0	13
9..........	3	40	9	1	38	7	0	30
10..........	16	19	10	0	20		0	37
						8	0	35
							0	24
						9	1	14
							0	33
Total........	87	290		6	275		2	326
Per cent of ♂♀.........	23.0			2.1			0.6	

Experiment XXX. From three sister females, three lines were reared, starting on July 18, 1910. One line was reared in a 0.02 per cent solution of the crude creatin in Great Bear spring water, one in a 0.025 per cent solution, the third in spring water alone. Table 32 shows the three pure lines in detail.

Experiment XXXI. This is a repetition of Experiment XXX, but with weaker solutions of creatin in both parts, a 0.01 per cent and a 0.015 per cent solution being used. In both of these experiments some of the eggs laid in the creatin solution did not hatch in the usual short time required in spring water. In such cases the creatin was diluted after the death of the parent, and many of the

TABLE 32

Showing the number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being bred in spring water, the other two in creatin solutions of different strengths.

SPRING WATER			0.02 PER CENT CREATIN			0.025 PER CENT CREATIN		
NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀
1	14	25	1	0	7	1	0	24
	2	30	2	0	11	2	0	28
	9	31	3	0	16		0	27
2	3	40	4	8	27	3	0	22
	4	15		0	3	4	0	17
3	16	19	5	0	29	5	0	16
	0	24		0	17	6	0	17
4	4	24	6	1	24		1	33
5	0	18		0	38	7	1	24
6	22	11	7	0	39	8	0	36
7	4	36		0	27	9	1	36
8	9	31	8	0	32	10	0	16
9	1	21		0	27	11	0	27
10	6	36	9	0	25		0	15*
11	7	14*		0	20	12	1	16*
			10	1	28*			
				0	18			
			11	0	17*			
Total	101	375		10	405		4	354
Per cent of ♂♀	21.2			2.4			1.1	

*Remainder of family not recorded.

eggs then hatched. Table 33 presents the details of the experiments.

In both of these tables the same result appears, that is, a lower percentage of male-producers in the creatin solution than in spring water, and a lower percentage in the stronger solution than in the weaker. The differences are as great as were those of the beef extract, and are too marked to leave any doubt regarding the influence of creatin.

EXPERIMENTS WITH EXTERNAL FACTORS

Those who have hitherto worked on the life-cycle of Hydatina have inclined strongly to one or the other of two extreme positions. On the one hand are those who hold that the proportion of male-producers at any time is a function of the external conditions then obtaining; on the other hand are those who believe that that proportion is determined by internal factors, uninfluenced by

TABLE 33

Showing the number of male- and female-producers in the progeny of three sister individuals of Hydatina senta, one line being reared in spring water, the other two in solutions of crude creatin of different strengths.

SPRING WATER			0.01 PER CENT CREATIN			0.015 PER CENT CREATIN		
NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀
1..........	4	24	1	1	13	1	2	14
2..........	0	18		0	25	2	0	28
3..........	22	11	2	2	46		0	18
4..........	4	36	3	3	39	3	0	19
5..........	9	31	4	0	43		0	18
6..........	1	21	5	0	25	4	0	28
7..........	6	36	6	0	22	5	0	36
8..........	7	14*		0	15	6	0	30
9..........	0	7*	7	0	30*	7	0	16*
				5	28*			
Total........	53	198		11	286		2	207
Per cent of ♂♀......	21.1			3.7			0.9	

*Remainder of family not recorded.

external agents. Clear expression is given to the latter view by Punnett ('06), who concluded that the only differences to be found in the proportion of male-producers were the differences in distinct pure lines, and that those differences depended on the character of the gametes uniting in the resting egg from which each pure line sprang. In my earlier work (Shull, '10b) it was shown that this conclusion of Punnett's did not hold in certain

cases there presented, where differences in the proportion of
male-producers were obtained within the same pure line. That did
not, however, exclude the possibility that such internal differences
do exist. In the hope of finding pure lines showing different
behavior with respect to the life cycle, search was made for
rotifers in different localities near New York. But this species
was nowhere found except at Grantwood, N. J. Eventually some
specimens were obtained from Baltimore, Md.

Experiment XXXII. A culture of Hydatina was obtained from
Baltimore through the courtesy of Prof. H. S. Jennings, and
brought to New York, on March 23, 1910, by Prof. T. H. Morgan.
The culture was labeled "Hydatina (Old Culture) Curtis Bay,
March 6." One of the females from this culture was isolated
March 23, and from her sixty successive generations were bred.
On the same day, a female was isolated from the pure line used
as control in Experiment XVIII, which was originally obtained
from New York, as described in that experiment. From this
female over fifty generations were reared. Both pure lines were
bred in Great Bear spring water, and the same food was used every
day for both. The conditions were kept as nearly alike as possible.
It was believed that any internal difference between the two lines
would be shown in so large a number of generations. In table
34 the two lines are compared in detail.

In the three months and a half through which this experiment
extended, the New York line produced only 11.1 per cent of
male-producers, the Baltimore line 18.5 per cent. Had such a
difference been obtained in any of the experiments with external
agents, it would have been taken as good evidence of a positive
effect of the environment. It can scarcely be regarded as other
than good evidence here. The difference is not due to some sudden
increase in the number of male-producers in one line without a
corresponding increase in the other line, for taken by and large
the periods of many male-producers occur about the same time
in both lines. Nor is the difference in the two percentages due
to the fact that the experiment was terminated at one time rather
than at another; for the experiment could have been brought to a
close at any time, even after a single generation, and the Balti-

TABLE 34

Showing the number of male- and female-producers in the progeny of two females of Hydatina senta, not related to one another, when reared under the same conditions. One female was collected at New York, the other at Baltimore.

NEW YORK PURE LINE				BALTIMORE PURE LINE			
NO. OF GENERA-TION.	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	DATE OF FIRST YOUNG	NO. OF ♂♀	NO. OF ♀♀
1............	Mar. 26	0	32	1	Mar. 25	8	22
2............	28	0	40	2	26	1	49
3............	29	7	40	3	28	18	37
4............	31	1	25	4	29	10	37
5............	Apr. 1	0	3	5	31	1	41
6............	3	0	19	6	Apr. 1	0	16
7............	5	1	50	7	3	0	39
8............	7	14	34	8	4	1	27
	6	0	2	9	6	23	27
9............	9	0	24	10	7	5	46
10............	11	0	19	11	9	7	42
11............	13	0	2*	12	11	1	34
12............	15	0	2*	13	12	0	2*
13............	16	0	1*	14	14	0	2*
14............	18	0	2*	15	15	0	2*
15............	20	0	2*	16	17	0	2*
16............	22	0	2*	17	19	0	2*
17............	24	0	4*	18	20	0	2*
18............	27	0	9	19	22	0	2*
19............	29	0	2	20	24	0	2*
	29	1	2	21	26	0	2*
	May 1	0	26	22	27	0	13
20............	2	0	7	23	29	4	39
	2	0	3	24	May 1	0	25
	1	0	2	25	2	5	41
	1	0	1	26	3	0	31
21............	3	0	14	27	5	4	44
	3	0	31	28	7	3	46
22............	6	1	34	29	9	5	37
23............	9	0	39	30	10	23	24
24............	11	1	8	31	12	18	25
25............	13	31	16	32	14	3	37
26............	15	8	30	33	16	6	41
27............	17	0	10	34	18	4	41
28............	19	1	27	35	19	22	26
29............	21	16	12	36	21	28	11
30............	23	0	31	37	23	13	24

TABLE 34—(CONTINUED)

NEW YORK PURE LINE					BALTIMORE PURE LINE		
NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♂	NO. OF ♀♀	NO. OF GENERATION	DATE OF FIRST YOUNG	NO. OF ♂♂	NO. OF ♀♀
31............	25	0	35	38	24	28	10
32............	26	1	38	39	26	6	35
33............	28	3	23	40	27	18	21
34............	30	0	2	41	29	29	7
	31	0	3	42	31	2	37
35............	June 1	0	33*	43	June 1	7	23
36............	3	1	18*	44	4	1	18*
37............	6	15	9*	45	6	0	2*
38............	8	1	1	46	8	0	27
.	12	10	14	47	9	9	30
39............	11	1	24		10	9	35
40............	14	4	20	48	13	2	39
41............	15	0	9	49	15	9	18
	16	0	11	50	16	12	29
42............	18	0	17·	51	18	0	38
	18	0	1	52	20	3	25
	19	0	3		20	0	26
43............	20	0	25	53	22	0	14
44............	21	5	22		22	0	21
45............	23	0	14	54	25	2	22
46............	24	0	1		25	0	8
	26	1	7		24	0	20
47............	25	6	12	55	26	14	28
48............	28	0	17	56	29	0	14
49............	30	0	6	57	30	1	23
50............	July 1	0	17	58	July 2	0	38
	2	0	2	59	4	0	18
51............	3	0	14	60	6	0	36
52............	5	0	1				
	6	1	32				
53............	7	0	1				
	7	0	6				
Total........	....	131	1045			365	1602
Per cent of ♂♀............		11.1				18.5	

*Remainder of family not recorded.

more line would have shown a higher percentage of male-producers than the New York line. Nor is this all. Half a dozen successive generations may be selected from any point in either line, and compared with the generations of the other line bred at the same time, and in nearly every case the percentage of male producers will be higher in the Baltimore line than in that from New York.

It is safe to say, therefore, that we have here two pure lines that differ from one another in a fairly constant manner, and the difference is an internal one. This ev dence seems on the face of it to support Punnett's contention that differences in pure lines might account for the results obtained by himself and previous workers. How far it supports his view, and whether the internal differences discovered are due to zygotic constitution, as Punnett suggested, are discussed elsewhere.

Such distinct pure lines having been found, it is important to known how the proportion of male-producers will behave if individuals of different pure lines are crossed. Will the proportion of male-producers in one of these lines behave as a dominant to that in the other, or will it exhibit no relation to Mendelian phenomena? To answer these and other questions, the several following experiments were performed.

Experiment XXXIII. A female from the eleventh generation of the Baltimore pure line of the preceding experiment, was paired April 12, 1910, with a male from the New York line. Resting eggs were laid by her on April 14 and several days following. These eggs were kept at room temperature, and from one of them, on April 23, there hatched a female. From this female the line in table 35 A, designated "Cross," was reared.

Another female of the Baltimore line was paired April 13, 1910, with a male from the New York line. One of the resting eggs from this female, having been kept at room temperature, hatched April 28. From this female offspring the line designated "Cross" in table 35 B, was bred. Each pure line designated "Cross" is compared with those parts of the parent (New York and Baltimore) pure lines which occurred simultaneously with it. All lines

in this experiment were reared under conditions as nearly alike as possible.

The objection may be raised that in making these crosses, the respective environments of the two pure lines were necessarily mixed, and that the pure line bred from one of the resting eggs

TABLE 35

Comparison of two distinct pure lines of Hydatina senta with another pure line derived from a cross between the first two, all being bred under like conditions. A and B are separate experiments.

EXPERIMENT	NEW YORK PURE LINE			PURE LINE FROM CROSS			BALTIMORE PURE LINE		
	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀
A	1	0	9	1	2	24	1	0	13
	2	0	2	2	15	37	2	4	39
		1	2	3	17	41	3	0	25
		0	26	4	4	47	4	5	41
	3	0	7	5	2	45	5	0	31
		0	3	6	22	29	6	4	44
		0	2	7	17	35	7	3	46
		0	1	8	7	47			
	4	0	14						
		0	31						
	5	1	34						
Total		2	131		86	305		16	239
Per cent of ♂♀	1.5			21.9			6.2		
B	1	0	2	1	5	37	1	4	39
		1	2	2	16	30	2	0	25
		0	26	3	3	41	3	5	41
	2	0	7	4	17	28	4	0	31
		0	3	5	12	42	5	4	44
		0	2	6	10	43	6	3	46
		0	1						
	3	0	14						
		0	31						
	4	1	34						
Total		2	122		63	221		16	226
Per cent of ♂♀	1.6			22.1			6.6		

was in a medium which always contained traces of this mixture. Precautions were taken to obviate any error in this regard. In the first place, it seemed likely that the extreme dilution to which any substance peculiar to either environment was subjected would have prevented that substance from exerting any appreciable influence. It must be remembered that the Baltimore pure line was bred for eleven generations under the same treatment as the New York line, before the crosses were made. The first female, and the females of each generation thereafter, were isolated in certainly not more than four drops of water. This was at once diluted to forty or more, or to one-tenth of the original concentration. In eleven generations, the concentration of any substance originally present would be only 10^{-11} times the concentration of that substance at the outset. Unless the substance had enormous powers of propagation, it would be negligible. But to preclude any possibility of error, an amount of water approximately equal to that in which the males were transferred in making the crosses, but containing no rotifers, was taken from each line and placed in the dishes of the other. So that from that time on both of the pure line were being reared in the same mixed environment as were the offspring of their cross. The same precaution was taken in each crossing experiment.

In both of these experiments (table 35 A and B) the pure line derived from the cross has a decidedly higher proportion of male-producers than did that part of either parent pure line which was bred at the same time. Moreover, it is higher than either of the parent lines produced as a whole, the entire series of generations in the New York line having produced but 11.1 per cent, the Baltimore line 18.5 per cent. It is to be noted that the resting eggs in these crosses were kept at room temperature, whereas many eggs in nature are subjected to continued low temperature. While no experiment of as great duration as a winter season was attempted, some eggs were kept for a brief time, as described in the next experiment, in an ice chest.

Experiment XXXIV. Two repetitions of the preceding experiment were made, except that the resting eggs obtained after crossing were kept for some days at a low temperature. Two females

TABLE 36

Comparison of two distinct pure lines of Hydatina senta with another pure line derived from a cross between the first two, the winter eggs of the cross having been kept for twelve days at low temperature. A and B are separate experiments.

EXPERIMENT	NEW YORK PURE LINE			PURE LINE FROM CROSS			BALTIMORE PURE LINE		
	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀
A.........	1	0	7	1	2	14	1	5	41
		0	3	2	3	22	2	0	31
		0	2	3	21	29	3	4	44
		0	1	4	4	49	4	3	46
	2	0	14	5	4	42	5	5	37
		0	31	6	2	50	6	23	24
	3	1	34	7	4	50	7	18	25
	4	0	39	8	3	50	8	3	37
	5	1	8	9	9	40	9	6	41
	6	31	16	10	41	9	10	4	41
	7	8	30	11	27	9	11	22	26
	8	0	10	12	0	18	12	28	11
	9	1	27						
	10	16	12						
Total		58	234		120	382		121	404
Per cent of ♂♀		19.8			23.9			23.0	
B.........	1	0	7	1	10	38	1	5	41
		0	3	2	0	27	2	0	31
		0	2	3	0	11	3	4	44
		0	1		0	23	4	3	46
	2	0	14	4	15	39	5	5	37
		0	31	5	8	47	6	23	24
	3	1	34	6	31	22	7	18	25
	4	0	39	7	26	28			
	5	1	8						
	6	31	16						
Total		33	155		90	235		58	248
Per cent of ♂♀		17.5			27.6			18.9	

from the Baltimore pure line were mated, April 13, 1910, with males from the New York pure line, and resting eggs obtained April 14 and several following days. These eggs were put, with the females, into an ice-chest at 8° to 10° C. on April 14, and left until April 26, when they were again brought to room temperature. On May 1, two females had hatched, one from each parent. From these two females, the lines designated "Cross" in table 36 A and B respectively, were reared. The parts of the parent (New York and Baltimore) pure lines reared at the same time are given for comparison.

Here again the pure line derived from the cross yields in each case more male-producers than do the parent lines, though the differences are small, especially in the first two experiments. The smallness of the difference is due, not to a lower proportion of male-producers derived from the cross than in Experiment XXXIII, but to a higher proportion in the parent lines than in the former experiment. The percentage of male-producers in the parent lines fluctuates considerably, and the delay in development due to low temperature caused the cross to be compared with the parent lines at a time of many male-producers in the latter. Obviously, an experiment of such extent is needed that the error due to these fluctuations may be reduced to a minimum. Such an experiment is the following one, in which the reciprocal cross is made.

Experiment XXXV. On May 14, 1910, a female of the New pure line was mated with a male of the Baltimore line, and resting eggs obtained May 15 and following days. The eggs were kept at room temperature until May 24, when one of them yielded a female. From her, the line in table 37 designated "Cross" was bred. This line is compared with those parts of the New York and Baltimore lines that occur simultaneously with it.

That large fluctuations in the proportion of male-producers in any line do not vitiate this experiment seems probable from the fact that the parts of the New York and Baltimore lines here given include approximately the same percentages of male-producers (10.8 and 15.9 respectively) as did those lines as a whole (11.1 and 18.5 respectively), as shown in table 34. If these figures

TABLE 37

Comparison of two distinct pure lines of Hydatina senta with another line derived from a cross between the first two, the cross being the reciprocal of that in tables 35 and 36.

NEW YORK PURE LINE			PURE LINE FROM CROSS			BALTIMORE PURE LINE		
NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERA-TION	NO. OF ♂♀	NO. OF ♀♀
1	1	38	1	15	40	1	6	35
2	3	23	2	37	23	2	18	21
3	0	2	3	13	21	3	29	7
	0	3	4	7	26	4	2	37
4	0	33*	5	30	19*	5	7	23
5	1	18*	6	2	17*	6	1	18*
6	15	9*	7	0	2*	7	0	2*
7	1	1	8	8	17	8	0	27
	10	14		6	22	9	9	30
8	1	24	9	22	29		9	35
9	4	20	10	35	19	10	2	39
10	0	9	11	22	12	11	9	18
	0	11	12	11	17	12	12	29
11	0	17	13	25	19	13	0	38
	0	1	14	19	26	14	3	25
	0	3	15	20	35		0	26
12	0	25	16	10	25	15	0	14
13	5	22	17	15	21		0	21
14	0	14	18	7	16	16	2	22
15	0	1	19	14	17		0	8
	1	7	20	17	41		0	20
16	6	12	21	8	45	17	14	28
17	0	17	22	1	45	18	0	14
18	0	6	23	31	29	19	1	23
19	0	17	24	9	13	20	0	38
	0	2	25	8	31	21	0	18
20	0	14	26	17	30	22	0	36
21	0	1	27	4	27			
	1	32						
22	0	1						
	0	6						
Total	49	403		413	684		124	652
Per cent of ♂♀	10.8			37.6			15.9	

* Remainder of family not recorded.

are trustworthy, as I am convinced they are, we have here the
remarkable result that the pure line from the cross yields not
only a greater percentage of male-producers than does either
parent line alone, but decidedly greater than the percentages of
both parent lines combined.

This result is so remarkable that it became of interest to breed
an individual of the pure line from the cross back to a member of
one of the parent lines. This was done in the following experiment.

Experiment XXXVI. Several females of the pure line derived
from the cross between a New York female and a Baltimore
male, in the last experiment, were mated with males of the New
York line on June 13 and 14. Of the winter eggs laid by these
females June 14 and following days, one hatched June 22. The
female from this egg gave rise to the line shown in the middle
column of table 38. By an extension of meaning, I shall speak of
the whole line derived from the cross between the New York
female and Baltimore male as F_1, and of the whole line derived
from the cross between an F_1 female and a New York male as F_2.
With F_2 are compared those parts of the parent (F_1 and New
York) lines which occurred simultaneously with it.

A comparison of table 37 with table 38 is most interesting. In
the former, where two rotifers in no way related to one another
were crossed, the F_1 gave rise to a line having a much higher pro-
portion of male-producers than either parent line; in the latter
table, where two related rotifers were crossed, the pure line result-
ing from the F_2 has a proportion of male-producers intermediate
between those of its two parent lines. It may also be remarked
that in the former experiment F_1 contained larger families and pro-
duced more generations in a given time than did either parent line;
whereas, in the latter experiment, the size of family in F_2 and the
number of generations in a given time is intermediate between
those of the two parent lines. If this is not an isolated case, it is
important.

TABLE 38

Comparison of New York line of Hydatina senta and of the line (F_1) derived from a cross between the New York and Baltimore lines, with another line (F_2) derived from a cross between the F_1 and New York lines.

NEW YORK PURE LINE			F_2			F_1		
NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀	NO. OF GENERATION	NO. OF ♂♀	NO. OF ♀♀
1	0	14	1	0	25	1	15	21
2	0	1	2	3	4	2	7	16
	1	7	3	1	38	3	14	17
3	6	12	4	19	13	4	17	41
4	0	17	5	4	20	5	8	45
5	0	6	6	8	34	6	1	45
6	0	17		0	13	7	31	29
	0	2	7	10	48	8	9	13
7	0	14	8	0	12	9	8	31
8	0	1	9	0	29	10	17	30
	1	32	10	2	31	11	4	27
9	0	1	11	0	22	12	8	23
	0	6	12	0	12	13	0	29
	0	11		0	7	14	0	12
	0	6	13	0	6	15	2	11
	0	1		0	7	16	4	28
	0	4	14	0	4	17	14	21
	0	1	15	0	12	18	15	21
	0	2	16	1	14	19	14	25
10	0	11	17	10	12			
	0	1						
	0	1						
	0	3						
	0	4						
11	0	4						
12	0	12						
	0	6						
13	0	2						
	0	4						
Total	8	203		58	363		188	485
Per cent of ♂♀	3.7		13.7			27.9		

DISCUSSION

Those who have studied parthenogenesis and sexual reproduction in Hydatina have drawn very different conclusions regarding the causes of the transition from one mode of reproduction to the other. Some have been impressed with the influence of external conditions in causing or preventing the inauguration of the sexual mode, others have held that internal factors are the chief if not the sole agents in this transition. Among the former were Maupas ('91) and Nussbaum ('97), to whom temperature and starvation, respectively, seemed sufficient to account for all the phenomena they observed. Punnett ('06), on the other hand, discarded both these external agents, and suggested that the factor which determined the proportion of male-producers (sexual females) is wholly internal, and is fixed at the time of fertilization. Whitney ('07), in his earlier paper, remained nearly neutral, merely stating that he found no evidence of external influence. He likewise found no evidence for the one internal agent, zygotic constitution, proposed by Punnett. But in a later paper (Whitney, '10) he implicitly ranges himself on the side of those who hold external factors accountable for all variations in the percentage of sexual forms. In my own article (Shull, '10b) all the evidence presented was in favor of external agents, though the possibility of internal factors was admitted.

The evidence presented in this and the earlier paper can hardly fail, I believe, to convince one that both external and internal agents are involved in the production of the life cycle. It has been shown that starvation, of itself, is probably not one of the external agents that have an influence on the proportion of male-producers; but any attempt to bring about starvation by experiment may make it seem to have an influence, because food can not be introduced in diminished amount without introducing diminished quantities of other things. We have found that differences in temperature may have an influence, as shown in this paper, but it is practically certain that this influence is an indirect one. If the effect of temperature were direct, it would be expected that its influence upon the proportion of male-producers would be

exerted always in the same direction. A comparison of tables 18 and 19 with table 20 shows that this is not the case. A lower temperature may result in either more or fewer male-producers. The results of the several experiments are not contradictory if we assume that the influence of temperature is indirect. Let us suppose that all the conditions, whether external or internal, with the exception of temperature, which exist at a given time, tend to produce at a given temperature either a higher or a lower proportion of male-producers than prevailed previous to that time. If at a lower temperature the response of the rotifers to other conditions is less than at higher temperatures, all the results here obtained find their explanation. In tables 18 and 19 we may suppose that conditions at room temperature tended to produce fewer male-producers; in the ice-chest, the rotifers did not respond to these conditions to the extent that they did at room temperature, and the result was more male-producers at the lower temperature. In table 20, it seems that a set of conditions was present at the end of January which tended to produce more male-producers, and that the low temperature of the ice-chest caused the rotifers to respond to these conditions to a smaller degree, the result being fewer male-producers at the lower temperature. This view encounters no difficulty in the fact that no difference in the the proportion of male-producers was obtained at a temperature of 20° and 24.°5 C., respectively; for if the temperature is sufficiently high that the rotifers may respond to other conditions to the greatest degree of which they are capable, it may make little difference which of several moderately high temperatures prevails.

In offering this explanation I have assumed that the response of the rotifers to both external and internal conditions may be modified by temperature. It is obviously possible to assume that the response to external conditions alone is so modified. In that case, the results shown in table 20 demand a set of external conditions which tended to increase the proportion of male-producers above that usually obtained from the same line in spring water. It is possible that such external agents exist. Whitney ('10) believes that there are chemical substances having such an effect.

So far, however, the chemical agents of which we have definite evidence, if added to spring water, all decrease, instead of increase, the proportion of male-producers. I have therefore preferred to assume, until more is known of the external agents, that the response of the rotifers to internal conditions may also be modified by temperature.

More effective than temperature in modifying the proportion of male-producers, are certain chemical substances, as a glance at tables 27 to 33 will show. Creatin has a remarkable effect in reducing the proportion of male-producers. Beef extract has an equal effect, but it is a mixture of many substances. Ammonium hydroxid and three ammonium salts, the chlorid, the carbonate, and the nitrate have a distinct effect, but not as marked as creatin or beef extract. Urea may almost certainly be put in the same class. All these substances tend to reduce the proportion of male-producers. Unlike temperature differences, the effect produced by these substances was in every case of the same sign. It is to be noted, however, that all of the experiments with a given substance were performed with a single pure line, whereas those with temperature included experiments with two distinct lines. This may or may not make a difference in the result.

To the above substances which have an undoubted influence may be added perhaps the degree of alkalinity. In several cases a greater alkalinity seemed to produce fewer male-producers, but the effects were slight and the results were not uniform.

Whitney ('10) in a recent communication is in substantial agreement with the author, in that he finds chemical substances responsible for considerable effects upon the life cycle. He has experimented with the manure solution used by me, and from these experiments, together with certain observations, he concludes that certain substances in the manure solution affect the proportion of male-producers. But in the details of the conclusion we differ. In my two former papers (Shull, '10a and '10b) I had expressed the conviction that certain substances *present* in the manure solution prevented the male-producers from appearing. Whitney believes that a certain substance may be present which causes male-producers to appear, and that it is only when

this substance is *absent* that female-producers alone occur. Inasmuch as I used old food cultures (manure solutions) without causing any epidemic of male-producers, Whitney holds that the substance which does cause male-producers to appear is a transitory product of decomposition of the manure, which is therefore present only in new solutions, not in old ones He states his point clearly (Whitney, '10, p. 348): "I would maintain that there seems to be a definite but transitory chemical substance produced in appreciable quantities in the decomposition processes in newly made horse manure cultures that can so act upon the parthenogenetic females as to cause them to produce sexual daughter females. When this substance is absent, no sexual females are ever produced, but only parthenogenetic females are produced * * * ." In these words Whitney clearly implies the belief that no internal agent in Hydatina ever tends to produce sexual females (male-producers) and that therefore whenever male-producers appear they are a manifestation of some external agent. I had assumed, on the contrary, that internal agents probably tended to cause some male-producers to appear, without the direct aid of any particular substances in the medium, and that the presence of certain substances prevented them.

In further support of the view that some substance causing male-producers to appear is present in new cultures but not in old ones, Whitney cites his own earlier findings (Whitney, '07) that the male-producers occurred predominantly in the earlier parts of the family. Since the food culture was new in the first part of the family and old in the latter part, one might expect that some chemical difference between old and new food cultures caused the male-producers to appear early in the family. That this evidence can not stand seems probable from data presented in the second of my papers (Shull, '10b), where it is shown, from a large number of families, that male-producers do not occur more abundantly in the first half of the family than in the last half.

Whitney has not found any specific substances which cause male-producers to appear. Search for them may well be successful and should certainly be made. But it is now certain that the *absence* of the substance which, he supposes, causes male-producers

to appear is not the only means by which they may be prevented from appearing. The presence of creatin, the presence of beef extract, the presence of certain ammonium compounds and of urea, all tend to prevent the occurrence of male-producers. These facts should not blind us, however, to the possibility, which Whitney points out, that other substances may cause male-producers to appear. As to the internal factors, which Whitney is inclined to discard in accounting for all male-producers, I have shown elsewhere that these internal agents exist; and I believe that we may attribute to internal agents, alone or in combination with external agents, some of the phenomena which have been held to indicate the presence of external agents alone.

Certain points of theoretical interest may be mentioned in connection with chemical substances. Inasmuch as the question was raised whether the action of temperature in altering the proportion of male-producers is direct, and was answered in the negative, it may also be asked whether that of chemical substances is direct. The chief evidence for supposing the influence of temperature to be indirect was the fact that it sometimes increased, sometimes diminished, the proportion of male-producers. Among chemical substances, on the other hand, with the exception of the degree of alkalinity, which produced differences too small to be of much value, the effect of each chemical was in every case tried of the same sign. This of itself would lead us to believe that the action of these substances is direct. But this is not necessary. The substances may, for example, induce a physiological state which is directly responsible for determining the mode of reproduction.

It may also be pointed out here that nothing in the evidence yet obtained shows whether these substances actually decide how events shall occur in a given cell, or whether, events having happened differently in two classes of cells, the substances in the medium merely decide which class may most rapidly and successfully develop.

The conclusion that certain external agents may modify the ratio of sexual to parthenogenetic females has, I believe, been

completely established. In like manner, it can no longer be doubted that, as intimated above, internal factors may exist which modify that ratio. This is of particular importance because of the tendency which has existed to attribute all the phenomena either to external factors alone or to internal factors alone. Little can yet be said regarding the nature of the internal differences. Punnett suggested that the ratio of male-producers in a pure line depends upon the character of the zygote from which the pure line springs, and the nature of the zygote in turn depends of course upon the nature of the gametes. Were it not for the possibility of modifying that ratio by external conditions, much in the experiments described in this paper might seem to support Punnett's view. When two unrelated individuals, coming from two pure lines which behaved differently with respect to the proportion of male-producers, were crossed, the zygote gave rise to a pure line which, when reared under the same conditions as the parent lines, yielded a higher proportion of male-producers than either parent line. The same result was obtained in every experiment of this kind. But when two rather closely related individuals, one from the F_1 line just mentioned, the other from one of the original parent lines (Experiment XXXVI), were mated, the zygote gave rise to a pure line having a proportion of male-producers intermediate between those of its two parent lines. If it had been found impossible to alter these results by employing different external agents in the different pure lines, we might perhaps be justified in saying that zygotic constitution is alone responsible for the ratio of male-producers. It was possible, however, to reverse the results. The F_1 pure line which was yielding more male-producers than either of its parent lines, was made, when reared in beef extract, to yield fewer male-producers than its parent lines. Other things being equal, some internal factor, *perhaps* zygotic constitution, causes different pure lines to yield different proportions of male-producers; but Punnett's assumption that zygotic constitution determines that proportion regardless of external conditions is not justified. There are no pure "strains" producing 40 per cent of male-producers or 2 per cent. A line producing 40 per cent can be made to yield 20 per cent as long as

the external conditions are properly selected; and any line with a not too high proportion of male-producers can be made to yield no male-producers. There is probably no such thing as a line which, under *all* circumstances, yields no male-producers. But even if we recognize that the internal factor is only one of several which together determine the proportion of male-producers, the internal agent may still not be zygotic constitution. It is quite possible that something, perhaps environment, may permanently modify the internal nature of a pure line, as Woltereck ('09) believes may occur in the daphnians. Such a modified internal nature could not be called zygotic constitution. Whether such a modification can occur in Hydatina is unknown, but it is conceivable; and so long as it is possible, we must not cling too tenaciously to zygotic constitution as the sole agent in producing the internal differences described.

It would be of great interest to know the process by which the internal factor, whatever it is, affects the life cycle. Does the cycle depend, internally, upon the quantity of something introduced by the gametes, or does it depend on segregable genes that combine and recombine in Mendelian fashion, or does it follow some rule not analogous to anything known in other animals? Furthermore, do any of these agents, external or internal, determine whether the cytological changes accompanying a change of the mode of reproduction shall occur in a given cell, or do they merely decide which of two already differentiated classes of cells shall go on and develop? These questions must for the present remain unanswered. There is no evidence on which to base an answer to the second question; while if we attempt to answer the first, all we can say is that if the internal agents work either quantitatively or in a Mendelian manner, the process is a complicated one. Results like those obtained from the crossing experiments with Hydatina have not been reported, so far as I know, from any other animal. A theory based on the few facts now known regarding this rotifer would be highly speculative and might have little value except as a pictorial representation to aid the memory. I prefer, therefore, to obtain more data before attempting to explain the results.

Whatever the process may be by which the life cycle is determined, it is clear that the cycle is affected by both external and internal agents. Neither one alone is responsible, we have to reckon with both. This is precisely the state of affairs which Woltereck ('09) finds in the daphnians, and which is confirmed by McClendon ('10) and in part by Papanicolau ('10). Both external and internal agents, according to these writers, influence the proportion of sexual forms. It will be profitable, therefore, to review briefly other groups in which there occur phenomena similar to those in Hydatina, and try to discover whether the recent findings in Hydatina will throw any possible light on these other groups. We may ignore such cases as occasional conjugation followed by long periods of simple fission without preceding conjugation, as in infusoria; or the alternating sexual and asexual modes of reproduction as exemplified by hydroids. These phenomena may or may not be related to that of alternating sexual and parthenogenetic reproduction in rotifers. This brief review may therefore be limited to the two groups of daphnians and aphids.

Regarding the daphnids, the question of alternating modes of reproduction, often spoken of as one of sex-determination just as in Hydatina, has been discussed by many workers, especially in Germany, and the views held have been more extreme than in the case of the rotifers. The first experimental work of any importance was that of Kurz ('74) who found that when the water in which a colony of daphnids lived was slowly evaporated, sexual forms appeared; but his experiment was not controlled. Schmankewitsch ('75) suggested that increase in the salt content of the water could cause sexual individuals to appear, just as he found it to produce morphological changes. Weismann ('79), after an extensive study of the problem, largely carried out in the field, rejected the findings of the two previous workers, and from his own observations drew the sweeping conclusion that no external agent in any way influences the occurrence of sexual individuals but that these are associated with certain generations, or better, with certain broods. It is further illustrative of Weismann's firm stand for internal determination, that he rejected the direct action of the external conditions as a means by which the associ-

ation of the sexual individuals with certain broods may have been brought about.

This conclusion of Weismann's was not openly challenged, so far as I can find, until Issakówitsch ('05) reported that he was able to increase the number of sexual individuals by starvation, and, indirectly, by low temperature. He went so far as to claim that a cycle, in Weismann's sense of the word, does not exist among daphnians. He recognized, however, that the longer a pure line was prevented from producing sexual forms, the greater was its tendency to do so, which may be a slight concession to Weismann's view. The conclusions of Issakówitsch were rejected by Keilhack ('06) and Strohl ('08) on insufficient grounds, it seems to me, while later workers have confirmed the conclusion that external agents are effective in determining the life cycle of certain daphnids. Langhans ('09) finds that the excretions of the daphnids themselves constitute such an agent; and Woltereck points out that different species and different local races behave differently in this regard, some species and pure lines responding readily to external conditions, others responding but slightly or having their cyle determined almost solely by internal factors. In some species, he finds, it is plain that both sets of agents are effective. McClendon ('10) likewise states that starvation, temperature differences, and excretions modify the life cycle, but he was unable wholly to exclude sexual forms in some cases. Papanicolau ('10) states that both internal and external conditions affect the life cycle, but not at all times in the cycle. In the two genera with which he worked, he recognized three periods in each cycle which differed in the susceptibility of the animals to external conditions. There is a purely parthenogenetic period, he says, comprising the first generation, counting from the resting egg, and the first broods of some later generations. The eggs laid in this period are, in the genus Moina, violet in color, and the females that hatch from them can not be made, by any external conditions, to show a sexual tendency. Another period, Papanicolau states, comprises the late broods of late generations, in which the females, which in Moina hatch from blue eggs, have a sexual tendency, and no external conditions can make them parthenogenetic. Between these is a period which

he calls the period of "transition from parthenogenesis to sexuality," characterized in Moina by violet-blue eggs, during which warmth induces parthenogenesis, and cold sexuality.

The phenomena in aphids have not been so thoroughly studied, probably because the external agents acting on them are not so easy to manipulate. The chief experiments that have been performed have consisted in keeping the aphids, and of course their food plants, in a warm place when cold weather advanced. Thus Kyber ('15) reared aphids parthenogenetically during a period of several years; and much more recently Slingerland ('93) has repeated the experiment with the same result. Slingerland's published data include 62 generations in 33 months, but I learned from Prof. Slingerland that the experiment was continued until more than ninety generations had been secured, without a single sexual form. Other experimenters and observers, some of them earlier even than Kyber, have agreed with these conclusions; but none have worked more extensively, hence it is needless to refer to them individually. Mention may also be made of the work on the corn root-aphis by Davis ('09), who has shown that the sexual forms are not associated with given generations. He was inclined to attribute the occurrence of sexual forms in his experiments to sea onal changes of temperature, though his experiments did not bear directly on this point. It is thus clear that external agents of some kind influence the cycle of some aphids. On the other hand, there are aphids in which the cycle is a closed one, the sexual females and males appearing in nature in a given generation, and attempts made to alter the cycle have so far proven fruitless. In the aphids, therefore, as in the rotifers and daphnians, it seems that both external and internal agents are effective in producing the life cycle.

This brief review of the work on parthenogenesis and sexual reproduction in daphnians and aphids is given merely that we may compare these two groups with the rotifers, and suggest that some light may be thrown upon the phenomena in the first two groups by our findings in the last Too little is as yet known of the internal factors to make a comparison profitable; but regarding external agents there is something to be said. The discovery that

chemical substances in the medium may influence the appearance
of sexual females among rotifers leads us to ask whether various
substances may not have a corresponding effect upon daphnids and
aphids. It is not necessary to suppose that the same substances
affect all these groups as affect one of them; nor will the effect
of any one substance necessarily be found to be the same in all
three groups. But it is certainly a pertinent question whether
chemical substances of some kind do not have an effect in one
direction or the other, either in producing sexual aphids and daph-
nids or in preventing their occurrence. Schmankewitsch ('75)
early suggested that the concentration of salts in the water might
influence cyclical phenomena among daphnians in nature. Kurz
('74) likewise named chemical change as one of several agents that
might have this effect. Their suggestions were rejected by Weis-
mann ('79), but Langhans ('09) has given evidence which he
believes shows that the excretions of the daphnians themselves
influence the proportion of sexual individuals. Papanicolau
('10) rejects the excretions as an agent affecting the cycle, citing
a short experiment of his own in support of his conclusion. One
of his colonies was reproducing only by parthenogenesis, another
was starting into a sexual period. He exchanged the water in the
two cultures, but at the end of four days the former colony was
still wholly parthenogenetic, the latter still partly sexual. Even
if we assume that the entrance of the sexual period in one of
these cultures was due to the accumulated excretions, it could
hardly be expected, it seems to me, that four days would suffice to
change the mode of reproduction in an animal in which a period
of two weeks or more elapses between one generation and the next.
Moreover, another phenomenon reported by Papanicolau may
perhaps be looked upon as supporting the view that chemical
substances affect the cycle. He finds that late broods of a given
female are more likely to contain sexual individuals than are early
broods of the same female. This reminds us of the similar phe-
nomenon in rotifers, where, however, the facts are just the reverse.
The late females of a family of Hydatina are more likely to pro-
duce parthenogenetic daughters (Shull, '10b) than are the early
females of the same family. The explanation suggested in my

paper for Hydatina, namely, that the accumulation of substances in the water towards the end of a family may be conducive to parthenogenesis, may possibly apply, *mutatis mutandis*, to daphnians. Until we are assured that the water into which a late brood was hatched was of the same chemical composition as at the beginning of the family, we must still consider the possibility that chemical substances, and not a factor connected with the age of the parent, are responsible for the difference in the sexual tendency in different parts of the family. In view of these considerations it is highly important that attempts be made to find single substances, or definitely known combinations of substances, which will modify the life cycle of daphnians.

Among the aphids the question is somewhat different. The medium in which these insects live is not suitable for conveying many chemical substances. Nevertheless, the character of their food plant may change chemically, and it is possible that this chemical change works large changes in the cycle of the aphids. Such chemical changes doubtless occur in autumn with the aging of the food plants. If the plants are reared under such conditions as fail to bring about the chemical changes which usually occur at that season, what influence may this not exert upon the life cycle of the insects?

I desire not to be misunderstood here because of certain terms I have used. If we are to ascribe the influence of external conditions on the cycle of the aphids to the chemical nature of their food plants, it might seem that this were a question of nutrition, whereas we have discarded starvation as a probable factor influencing the cycle in Hydatina. It need only be pointed out in this connection that those who have advocated the view that nutrition influences any of these alternating cycles have referred to quantity, not chemical quality, of food. It may even be suggested by some that, in rotifers, where chemical substances have been proven to be effective agents, these substances act only indirectly by stimulating or otherwise affecting nutrition. Even if this be true, the real agent is not quantity of food available, as was formerly held to be the case.

In the light of these experiments on rotifers, it is obvious, it seems to me, that the assumed influence of temperature and nutrition upon the life cycle of the aphids and daphnians must be re-examined, to discover whether some of the phenomena observed in those groups and attributed to the two factors named, may not rather be due to the action of chemical substances.

SUMMARY

At an average temperature of about 20° C and 24°.5 C., respectively, two pure lines of rotifers yielded practically the same proportion of male-producers.

At an average temperature of 10° C. the rotifers yielded in several cases a decidedly higher proportion of male-producers than at 20° C., but in one instance a lower proportion. This may be taken to indicate that the influence of temperature is indirect.

A solution of horse-manure may wholly prevent male-producers from appearing. Boiling such a manure solution does not diminish its effect, neither does drying and redissolving it.

The substance in manure solution which prevents male-producers from appearing is apparently not soluble in ether nor absolute alcohol.

The brown colored substance of manure solutions probably does not affect the proportion of male-producers.

A higher degree of alkalinity seems to result sometimes in fewer male-producers than does a lower degree of alkalinity; but the differences obtained were slight and the results were not uniform.

A solution of urea tends to reduce the proportion of male-producers. Other substances having the same effect are ammonium hydroxid and three ammonium salts, the chlorid, the carbonate, and the nitrate.

Beef extract and creatin solutions greatly reduce the proportion of male-producers.

Two pure lines obtained from widely separated localities, and hence not related to one another, were found to yield a constantly different proportion of male-producers, even though external conditions were the same for both.

When a member of one of the above pure lines was mated with a member of the other pure line, the zygotes gave rise to pure lines having in every case a higher proportion of male-producers than had either parent pure line. This was true of every zygote tested, regardless of which pure line had furnished the female parent.

When a member of one of the pure lines derived from the crosses above was mated with a member of one of the original parent lines, the zygote gave rise to a pure line having a proportion of male-producers intermediate between those of the two parent lines of this zygote.

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THE PHOTIC REACTIONS OF SARCOPHAGID FLIES, ESPECIALLY LUCILIA CAESAR LINN. AND CALLIPHORA VOMITORIA LINN[1]

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TWENTY-FIVE FIGURES

CONTENTS

[1] The classification of the Sarcophagidae as here used is based on Girschner's ('96) system. See also Coquillett ('01).

I. GENERAL INTRODUCTION

1. Historical

Though the blow-flies (including Lucilia caesar) have been the object of study in early times, the first published observations, to the writer's knowledge, relative to the larval reactions to light were given by Pouchet ('72). This writer reports certain observations and experiments by which he demonstrated the sensitiveness of these larvae to light; he observed that larvae that were feeding in a mass of old hair and other slaughter-house refuse, came to the surface at night and crept back into the mass at daylight, or when artificial light was thrown on them. To prove that heat had no part in the reaction, Pouchet placed a vessel containing water heated to about 80° [C] on top of the heap of refuse, with the result that the larvae did not move away from the vessel as from the light, but on the contrary tended to collect in the vicinity of the warm body. Having satisfied himself experimentally that the larvae are sensitive to *light*, he then set about discovering what organs are affected, and also whether the quality and intensity of the light have any effect. Eristalis tenax and Lucilia caesar were used for the investigation and it was shown that the larvae were not only sensitive to light, a fact which Lowne ('90, '92 p. 71) also observed, but that they responded to the direction of the ray, Pouchet moreover showed that the reaction to light was immediate. He proposed the term "Actinaesthesie" to designate the property which dipterous larvae without eyes, but responsive to light possess. By means of this sense they become receptive to the intensity and direction of luminous radiations. Besides discussing the tracks made by the larvae, he took up the question of the age at which the larvae begin to show sensitiveness to light and it was shown that they avoided light immediately upon hatching, but that at that time they were not able to react to its direction. This latter reaction grows progressively as the individual gets older but is not fully developed until the larva has completed its growth. Pouchet concludes that actinaesthesia depends upon the ocular buds (the embryonic eyes, so-called), that the light impinging at different angles on the

surfaces of these buds, gives the animal the sense of direction, and that it is possible in consequence that the vision of the perfect insect may be reduced to this simple faculty.

Loeb ('90) in his studies on heliotropism worked upon the larva of Musca vomitoria because of its negative reaction to light and its eyeless condition. He found that the extreme anterior portion of the larva is highly sensitive to light.

In a recent paper by Holmes ('05a) the phototactic movements of individual fly-larvae (blow-fly), together with those of other species receive attention. In the words of the author (p. 98): "No one has attempted to work out in detail the exact mode of response in any of these forms, although the fact of their orientation to the direction of the rays of light has been described by several different observers." The conclusions which Holmes (p. 105) reaches in reference to the fly-larvae are as follows: "In the animals here described there is, so far as I can discover, no forced orientation brought about by the unequal stimulation of the two sides of the body, but an orientation is produced indirectly by following up those chance movements which bring respite from the stimulus. I do not deny that there may be an orientating tendency of the usual kind, but if there is, it plays only a subordinate rôle in directing the movements of the animal."

The writer (Herms, '07) in a preliminary study of the tropisms of fly-larvae as a factor in food habits and migration (pp. 77–82) pointed out a *positive* reaction of the larvae of Lucilia caesar. This reaction was further discussed in a paper read at the Seventh International Zoölogical Congress (Herms '11).

2. Anatomical

It is a generally known fact that fly-larvae have no obvious, external visual organs, and this fact alone has made interesting the observations relative to their locomotion and accommodation to external stimuli. Weismann ('64) and Lowne ('90–92, '93–95) have treated quite exhaustively the anatomy of the 'blow-fly' through all stages of its development, and it is largely on the work of these two authors that the following statements are based.

The only special stuctures which might be considered as light receptors are the unpigmented eye-like organs described by Lowne ('90–92, pp. 71–72) at the extremity of the maxilla. These organs have never to my knowledge been considered as visual organs, and Weismann and Lowne evidently do not consider them as such.

Concerning external segmentation Lowne ('90–92, p. 33) states that "Although it would appear at first sight perfectly easy to count these segments, authors are by no means agreed as to their number." The difference of opinion is due to invagination at the anterior end and fusion at the posterior end. There are in the two species considered in this paper, twelve obvious segments, and when reference is made to the head-segment the first of these twelve is meant. This first segment according to Lowne ('90–92, p. 35) is composed of three metameres followed by three thoracic and nine abdominal segments. Since there is only one visible cephalic segment (distinguished by the maxillary hooks) and since the last segment is a fusion of two, there remains the obvious number twelve, not including the invaginated segment of Newport (Lowne '90–92, p. 34) between the head and the first thoracic segment. "When the maggot emerges from the egg (Lowne, '90–92, p. 2) the parts destined to form the head in the perfect insect are found deeply invaginated, and lie far back in the thoracic region in front of the highly concentrated nervous system.

"The central nervous system in the larva of the cycloraphic Diptera generally (Lowne, '90–92, pp. 67–68) differs widely from that of other insects in the close concentration of all the ganglia in a single complex centre, which consists in part of the differentiated nervous system of the larva, and in part of embryonic structures destined to form the nerve centers in the nymph. Although these parts are intermixed in a complex manner, the cellular elements of each are distinctly recognizable, and those which are active in the larva undergo degeneration, like the rest of the larval tissues, whilst those which are embryonic in type undergo evolutions during the formation of the nymph." The central nervous system which is devoid of the usual ventral nerve-cord is comparatively short (Weismann, '64, p. 205), about one-twentieth of the length of the body; in a larva 1.3 cm. long, it measures only 0.74 mm.

"In the larva of the blow-fly the optic disc (Lowne, '93–95, p. 545) is connected with the central nervous system by the optic stalk, Weismann's 'Nervenstiel,' so that it has the form of a mushroom, the optic stalk forming its stem. The relation of the optic stalk with the central nervous system, indicates undoubtedly that it is a rudimentary optic nerve; it consists chiefly of neuroblastic cells and their processes, and exhibits a distinct central cavity. It expands beneath the optic disc, and may represent a rudimentary retina: and it is covered by a thin layer of parablastic cells, its peritoneal covering. In this stage the eye disc differs in no respect from the other imaginal discs, so that there is no reason to suppose that its nervous stalk possesses any functional activity as a nerve of sight. The disc is neither pigmented nor has it any special end organs."

The "great hooks" of the larva are described by Lowne ('90–92, pp. 40–41) as "uncinate processes of the cuticular layer of the integument." "When at rest they are retracted within special cavities in the maxillae, which do not communicate with the mouth. They are the retractile claws of the maxillae,   .   .   .

They are used as organs of locomotion and probably assist in the disintegration of the flesh in which the larva burrows."

3. The natural history of the sarcophagid flies

The principal interest in members of this group at first centered on their usefulness as scavengers of the Lake Erie beach débris which is composed largely of dead fish washed up periodically in large numbers by the surf. (Herms, '07, p. 74). Observations were carried on during the summer months (July and August) at the Lake Laboratory,[2] Sandusky, Ohio, for five successive seasons and during the autumn and winter months of 1907–08 at the Zoölogical Laboratory of the Museum of Comparative

Zoölogy[3] at Harvard University, Cambridge, Mass. The present paper was practically completed in June, 1908, though not presented for publication till July, 1910.

The species upon which observations were made at Sandusky are Lucilia caesar Linn., Compsomyia macellaria Fabr., Sarcophaga sarraceniae Riley, and Sarcophaga assidua Walker. Where there was such an abundance of food as at the Lake Laboratory one might also have expected to find Calliphora vomitoria Linn., but this species was rarely seen on Cedar Point in the neighborhood of the Laboratory, during the period of observation.

The very uniform surroundings and constant food supply obtaining during July and August at Cedar Point (Herms, '07, p. 74), together with the great warmth at this time of the year resulted in a rapid sequence of broods and a remarkably regular life history for each species (Herms, '07, p. 54). Irregularity is rather the rule for situations removed from the beach (Herms '07, p. 76), and this was particularly noticeable at Cambridge, where, moreover, late autumn and winter conditions had to be dealt with. Here, however, the flies were reared in a room where the temperature was kept artificially high and by careful attention to the kind and quantity of food, a continuous colony was established. Fish was invariably given as food for the larvae, while the flies were kept well supplied with water, occasionally sweetened with sugar. Notwithstanding the extreme care, the same regularity for the various stages in the life history as observed at Cedar Point was not secured in Cambridge.

For experimental purposes the two species which deposit eggs (Lucilia caesar and Calliphora vomitoria) were selected so that the time of hatching could be recorded. The former was used at Cedar Point and both species in Cambridge. It may be noted here incidentally that Compsomyia macellaria, the screw-worm fly, deposits eggs in the Southern States (Osborn, '96, p. 131),

though it is viviparous northward. This difference in habit has been pointed out by Portschinsky ('85) for Musca corvina, which always deposits eggs in the neighborhood of St. Petersburg, though in southern Russia it is usually viviparous in the summer (see also Osten Sacken,'87).

For convenience in reference I shall designate the stages[4] into which the life-history of the flies in question naturally fall, as follows:

a. The *egg stage*, representing the period from the extrusion of the egg to the time of its hatching.

b. The *larval stage*, extending from the time of hatching until pupation. This stage is sharply divided into two periods by the *migration* of the larva from the flesh upon which it has been feeding, to bury itself in the earth or among debris. These two periods I shall designate as (1) the *feeding period* and (2) the *prepupal period*, the latter being usually referred to as the resting stage. From the standpoint of habits these two periods are quite distinct and it is therefore not sufficient to refer merely to the time of infancy as the larval stage. The first few days after hatching are distinctly a period of excessive feeding (Herms '07, pp. 57–67 on normal growth), and the days immediately preceding pupation are not strictly a resting period, since the larvae are quite active under conditions to be described later. However, they do not feed during this time even when after weeks of starvation they are given the opportunity. Hence it seems preferable to refer to this simply as the prepupal period. This period, of all periods of the life-history of these insects, is subject to the greatest variation in length due mainly to changes in temperature and moisture (Herms, '07, p. 52).

c. The *pupal stage* which is distinctly a quiescent stage so far as locomotion is concerned. Before the pupal case has become opaque, if light is thrown on the animal, movements of the internal organs can clearly be seen. Muscular connection with the case, is however, soon severed and locomotion is no longer possible. The disintegration of the larval tissues takes place ordinarily in from

[4]For the duration of these stages the reader is referred to table 1.

twenty-four to thirty-six hours after pupation, and on the third day reorganization from the imaginal discs is already in rapid progress.

d. The *imago stage*, which is reached on the emergence of the full-grown fly from the pupa case. At this stage the reproductive products are not fully mature, except possibly in the male. In the female the ova are not ripe for from nine to twenty-one days in Lucilia caesar and from twelve to thirty-six days in Calliphora vomitoria. This difference in the time required for ripening the reproductive products influences the success with which breeding may be carried on *inter se* in the same brood.

e. The *adult stage*, which may be said to be that part of the imago's existence that is characterized by sexual maturity. In the male imago this is arrived at after perhaps a few hours and in the female after from nine to thirty-six days.

The question has frequently been asked: How long does a fly live? The following obervations may aid in answering this question. It should be remembered, of course, that flies may hibernate in the early imago or even the adult stage for several months, though they may at any time be rendered active by exceptional warmth. The writer saw specimens of Lucilia caesar active out of doors in Cambridge in the middle of January. This hibernation causes the life of a fly to be greatly prolonged, possibly to six months, but the life time of an individual should be considered primarily from the standpoint of its *active* life. My records show considerable variation in this stage for both males and females of the two observed species. The observations as recorded do not warrant the belief that the female fly has a longer lifetime than the male. It may be seen from table 1 that the usual length of life of the imago is about thirty days, regardless of sex. The oldest individual (Calliphora vomitoria) lived as an imago 63 days.

TABLE 1

Showing the duration of life of (a)Lucilia caesar and (b) Calliphora vomitoria

STAGES	LUCILIA CAESAR AT CEDAR POINT.			LUCILIA CEASAR AT CAMBRIDGE.			CALLIPHORA VOMITORIA AT CAMBRIDGE.		
	Short-est.	Modal.	Long-est.	Shortest.	Modal.	Longest.	Shortest.	Modal.	Longest.
Egg	8hrs.	18±1hrs.	24hrs.	24±1hrs.	24±1 hrs	48±1hrs.	24±1hrs.	24±1hrs.	48±1hrs.
Feeding	2 ds.	2½ ds.	3 ds.	3 ds.	5±1 ds.	7 ds.	3 ds.	6±1 ds.	9 ds.
Prepupal	2 ds.	3 ds.	4 ds.	4 ds.	6±1 ds.	85 ds.	2 ds.	4±1 ds.	7 ds.
Pupal	8 ds.	8 ds.	8 ds.	8 ds.	12±1 ds.	34 ds.	10 ds.	11±1 ds.	17 ds.
Adult (incl. imago).	?	?	?	1–da.	30±1 ds.	45 ds.	1–da.	34±1 ds.	63 ds.
Totals	?	?	?	17 ds.	54±ds.	173 ds.	17 ds.	56±ds.	98 ds.

II. AGGREGATED LARVAE

1. Introduction

A lake beach, such as that of Lake Erie at Cedar Point with its quantity of dead fish cast up at fairly regular intervals throughout the entire summer, affords a rare opportunity for the study of sarcophagid fly-larvae in their aggregated reactions, and the ease with which the animals can be reared in the laboratory under practically normal conditions is also favorable for study. A combination of field and laboratory observation affords in the writer's estimation, the most satisfactory basis for experimental work. The normal environment and normal behavior of the organism should have the experimentor's closest attention, in order to aid in the correct interpretation of the phenomena observed under experimental conditions.

Much of an earlier paper already referred to (Herms '07) is taken up with the consideration of field observations. The present paper is concerned largely with one factor in the behavior of sarcophagid fly-larvae, namely light.

It has been pointed out earlier that the migrated larvae were found to be active at night, and that migration from the carcass took place openly at night and concealed during the day, *i.e.*,

if the feeding period ended during the daytime the larvae escaped by means of an opening eaten through the side of the dead fish nearest the earth, and thus made their way into the sand beneath, without much exposure to daylight. This behavior was supposed at first to be due to the heated condition of the sand during the day, since on very cloudy days when the sand was cool, the open migration was occasionally observed. The concealed migration is of much value to the larvae, since they are thus protected quite largely from the ravages of birds, though the sandpipers may be found busily patrolling the beach in the dead of night, very probably devouring numbers of larvae, as evidenced by their tracks about fish carcases from which the larvae had migrated. On very cloudy days I have observed these birds feeding on the chance migrating individuals. Furthermore, during the day the sand of the beach usually becomes extremely hot (commonly 78° C. just beneath the surface) and larvae attempting to migrate at such a time are literally baked, as has been demonstrated (Herms, '07, p. 52). The concealed method of migration prevents such fatalities to a considerable extent.

It was also frequently oberved that the larvae feed in far more unprotected positions with regard to light during the night than is the rule during the day; even occasional short excursions away from the flesh are taken at this time as the migration period draws near.

There remains to be considered then the range of intensity through which these larvae react and the manner of their reaction. The positive reaction, as already mentioned, needs also to be considered in further detail.

2. Observations and experiments

For experimental purposes at the Lake Laboratory, eggs were collected during the previous day in order that the larvae might hatch under observation the following morning. On hatching, the larvae immediately crawl away from the egg shells into darker, less exposed situations and frequently underneath the fish, where there is moisture. Moisture is an important factor in the move-

ments of the larvae, especially at this stage. Here they collect, not always in one mass, but usually so. While certain experiments were being carried on it was noticed that the larvae responded very definitely to ordinary lamplight. This particular response was not negative but positive; that is the larvae left the flesh upon which they were feeding and moved toward the light, taking up a position finally at a point as near as possible to the source of light. This peculiar reaction was first observed on the evening of June 30, 1905, and is more fully described as follows:

Experiment 1. June 30, 1905. This was an experiment on larvae of Lucilia caesar, which under experimental conditions had crawled into a small vial 12.7 cm. in length, containing a piece of fish weighing about one gram. Within ten minutes after lighting the lamp the larvae, (between sixteen and eighteen hours old), numbering thirty-six, were noticed to leave the flesh, crawling rapidly toward that part of the vial nearest the light. This took them 4.5 cm. away from the food; changing the angle between the vial and the light, or rolling the vial over always resulted in a readjustment on the part of the individual animals. Moving the lamp from one side of the vial to the other likewise resulted in readjustment. Gradually increasing the distance between the vial and the light resulted in a final return to the food when a maximum of four metres was reached. On bringing the lamp near again, the larvae once more left the meat when the light was three metres distant. The experiment was repreated several times with like results, not only with the same larvae, but with different sets of individuals.

The experiment was tried one year later with the following results, which are practically identical with the results of Experiment 1.

Experiment 2. July 24, 1906, 8:20 p.m. A covered glass vessel (containing larvae feeding on fish) was placed in position on a table with a lamp 40 cm. distant.

8:30. The larvae were moving away from the flesh.

8:35. They had collected at the point within the vessel which was nearest to the light, at a distance of 2 to 5.2 cm. from the flesh.

8:50.   The lamp was placed about one metre away, causing an immediate retreat of the larvae toward the flesh.

8:55.   They were again collecting at this time as at 8.35.

8:56.   The lamp was moved to a distance of two metres.

8:57.   The entire mass was moving toward the flesh and away from the light.

8:58.   The larvae were again drawing nearer to the light.

9:00.   Moving the lamp to a distance of three metres, resulted as before.   In this case it was necessary to place the lamp somewhat higher than previously, to which change the larvae responded.

9:04.   The lamp was moved to a distance of three and one-half metres.

9:06.   The larvae were retreating toward the flesh slowly and in a rather scattered manner.

9:10.   The mass was very close to the flesh.

9:11.   The larvae were moving away from the flesh, but in two groups: one massed, the other rather scattered.

9:12.   The scattered larvae had assembled and the individuals in the mass were moving toward them.   This new aggregate actually proved to have collected at the point nearest the light.

9:16.   The lamp was moved to four metres.

9:25.   Out of about 200 larvae in the vessel all but about a dozen had returned to the flesh on the side away from the light; there are usually some stragglers under weak illumination.

9:27.   The lamp was placed once more at three metres distance.   The response was very slow.

9:38.   The entire mass of larvae had again assembled just at the edge of the flesh.   (The experiment was interrupted).

Though only an ordinary kerosene lamp with a small (no. 1) burner was used in these experiments and no intensities were calculated the results are nevertheless interesting.   It may be said with assurance that the larvae when aggregated, react positively to lamplight; even at a very low intensity; and that the experiments afford examples of positive phototaxis acting more strongly than normal positive chemotaxis, provided the experiments cited in my earlier paper (Herms, '07, pp. 78–79) are

accepted as conclusive evidence for chemotaxis with regard to food conditions (fish).

A reaction by half grown larvae of Lucilia caesar was noted and described by Pouchet ('72, pp. 133–134) which is undoubtedly analogous to the positive reactions under consideration. The reaction is referred to as a "Perversion provoquée de la sensation lumineuse."

On the beach the entire mass of larvae can be caused to collect at a point on the fish nearest the light from an acetylene lantern but usually in touch with the fish through other individuals. The behavior of the larvae on the beach depends on their age, as was also observed in the laboratory, *i.e.*, older individuals react more slowly and sooner or later always return to the flesh, while the younger may remain away from the flesh indefinitely. It should be noted here that the feeding animals react in the manner described throughout the feeding period, but not to the same degree. Very young larvae, from hatching to several hours thereafter, do not react thus in an appreciable manner; the ages between twelve and forty-eight hours are evidently influenced most, for after two days the positive response becomes feebler until migration, when the larvae are decidedly negative to any kind of light. To strong diffuse daylight and sunlight they are *always* negative.

Experiment 3. July 28, 1906, 9:00 p.m. Larvae between fifty-five and sixty hours old, still feeding within the body of a fish on the beach, were drawn toward the light in an aggregated condition. The body of the fish had been cut open along the abdomen and through this opening the larvae crawled out upon the sand and along the edge of the fish toward the point of strongest illumination, accumulating in that region. Moving the lamp either toward the head or toward the tail of the fish caused the mass to follow. The response was quite rapid, not more than five to six minutes being required to shift the position of the entire mass over a space of ten to twelve centimetres. The lamp was left in place twenty-five minutes; on returning at the end of that time, it was found that the animals had all retreated to the original position within the fish.

The surging out of the mass of larvae from the body of the fish, might have been supposed to be due to the disturbance caused by cutting open the carcass along the abdomen, but it should be borne in mind that when they were disturbed, as was shown in Experiment 2 (in that case by a mere shifting of the lamp), they invariably moved away from the source of light as is also shown in the experiments to follow.

Experiment 4. August 14, 1906, 8:30 p.m. This experiment was made on larvae from twelve to fourteen hours old, and showed that a mass of larvae about 4 cm. in diameter could shift its position 2.5 cm. in about one minute. In this case the larvae travelled *away* from the fish and toward the light at a comparatively rapid rate, The experiment was necessarily closed after five minutes trial.

This experiment differs from the preceding one in that the larvae wholly left the fish and moved toward the light, while in the former (Experiment 3) the larvae remained more or less in touch with the body of the fish and were merely massed in a strongly illuminated region.

The following experiments may be cited as further illustration of the positive reaction of the fly-larvae. While colored glass was used in some of these experiments the results are not to be considered as decisive for their color reactions, though this may be an approximation to the actual conditions. The glass was not tested for its efficiency as a light filter by any rigid method; ruby glass however, may be considered reasonably safe for red when of uniform thickness; the blue glass on the other hand is pretty surely unreliable. The rather remarkable results obtained by means of the spectrum are interesting and uniform.

For experimental purposes the animals were retained in a glass box (10x12.5x17.5 cm.) constructed of clear photographic plates. Usually from 200 to 500 larvae were used for each experiment, and their food consisted in all cases of fish. As a rule only a portion of the fish upon which the eggs had been deposited was taken as food for the larvae. A "Model C" automatic acetylene lantern was used, which gave a fairly satisfactory light in quality, though somewhat variable in intensity. The prism was made by carefully cementing together three plates of glass (12.5 cm.x17.5

cm.) on a triangular base. This vessel was then either filled with pure water or a saturated aqueous solution of alum. Sunlight was reflected on the prism by means of a small miror. The experiments were carried on in a darkened room, usually with controls and frequent repetitions.

Experiment 5. July 27, 1907, 7.30 p.m. About 500 larvae 33 to 35 hours old were in a mass on the flesh.

7:52. The acetylene gas-lamp was placed in front of the box, (12 to 14 cm. from the mass) causing the usual scattering of the individuals toward the farther side of the box. In two minutes the larvae were moving in a stream toward the light, massing directly in front of the flame. By 8:00 o'clock (8 minutes) all but a few stragglers had collected on the glass front as high as the flame.

8:00. A red glass was placed in front of the lamp, resulting in no apparent disturbance.

8:05. The red glass was replaced gradually by a blue glass, which also caused no noticeable disturbance.

8:10. The larger number of larvae had crawled to the top of the glass front, while the remainer had returned to the flesh. About a dozen were found immediately opposite the flame, but these were moving either up or down the glass front.

8:15. The red glass was placed in position again.

8.20. About half of the larvae had collected directly in front of the light when the lamp was taken away leaving them in darkness.

8:35. The individuals had practically all returned to the flesh.

9:15. After leaving the larvae in darkness almost an hour, the lamp was once more placed in position with the blue glass.

9:30. The animals, following the same tactics as before, had crawled up the side of the box to the top (16 cm. of travelled distance from the flesh).

Experiment 6. July 27, 1907, 2:20 p.m. About 500 larvae, aged 28–30 hours, were contained in the glass box and massed on the flesh. The light in this case was conducted through the prism already described and used at a distance of three metres from its source. Throwing the blue end of the spectrum on the mass

caused individuals to move away from the light to the opposite side and underneath the fish.

2:40. By this time all the larvae had crawled away and were beneath the flesh, and individuals did not return to the light side which would have been done in this time were such a reaction to take place. Now the red and orange bands were cast on the flesh. In five minutes the larvae began moving toward the light, taking a diagonal course away from the flesh into and through the yellow band.

3:00. A mass of at least 150 larvae had collected in the yellow band with many coming from the flesh and some returning toward it. The collected larvae were 3.5 to 4 cm. distance from the flesh and many of them had crawled 2 to 3 cm. up the face of the box. In going toward the light the larvae followed a diagonal path from the flesh which took them out of the red and orange bands into the yellow, across which they travelled to the green band. When the green band was encountered they changed their course by taking the path on the border of the yellow, following this course to the edge of the box nearest the source of light. (The change in direction indicated could not be due to unequal distribution of moist surface, since the entire bottom was well "smeared" with fluids from the flesh.) This behavior massed the larvae in the yellow band nearest the green, into the edge of which this aggregation naturally grew, but the collecting here was compensated for by a movement away from the green into the more intense yellow portions, never through the green into the blue end. The larvae that left the mass by crawling along the inner edge of the box, probably a thigmotactic response, encountered the orange, which was followed back to the flesh. Some individuals also took this latter route toward the flesh, and some returned by the route usually taken *to* the flesh. Not more than 60 to 75 larvae were separated from the general mass at any one time.

3:45. The violet end of the spectrum was thrown on the mass for a few minutes, causing a general movement away from the source of light. The larvae were then left in semi-darkness for an hour during which time they remained in the same general position as during the earlier part of the experiment.

7:30. The larvae had all returned to the flesh where they were massed. Exactly when this return took place is not known.

Experiment 7. July 24, 1907, 2.35 p.m. A total of about 350 migrated larvae were in the box. Of these an aggregation of 75 to 80 had collected in the upper corner of one end, and a larger mass was collected in a lower corner on the end opposite and diagonal from the former aggregation away from the source of light as far as possible under the conditions. Throwing the spectrum on the larvae put the smaller mass in the red and orange bands, and the larger in the blue end, practically under the ultra violet. After ten minutes in this position no perceptible change in distribution for the general masses was noticeable.

2:45. At this time the box was turned end for end which placed the larger aggregation under the red and nearest the source of light and the smaller under the blue. A very slow shifting of both aggregations began, the larger mass gradually working away from the front of the box to the farther side taking a diagonal course, until that side was reached at 3:30. The animals in the violet gradually moved out of this color through the blue into the yellow and orange. At 3.30 they were distributed as follows: 9 on the bottom in the red, 16 on the face toward the light, 12 on the back away from the light (in moving about some larvae scattered so that there was a total of about 75 larvae moving slowly at random in the entire blue end); all the remaining individuals were in the red end, the mass lying in the yellow and orange bands.

3:45. Changing the box end for end again resulted in a rapid and most marked movement away from the source of light, out of the blue and green and into the yellow, orange and red, massing under the yellow and orange as before.

Experiment 8. July 25, 1907, 2:35 p.m. The migrated larvae that had been used in Experiment 7, had collected with few exceptions in a corner farthest away from the daylight. Casting the spectrum on the box with the mass of larvae under the orange and red caused comparatively little disturbance. Such individuals as had collected in the opposite corner now in the violet, crawled

out of range in a few minutes, excepting a few (6) which were crawling about at random in the blue and green.

2:45. No change of position on the part of the aggregated larvae had taken place. (The migrated individuals come to rest usually in a mass, when retained in a receptacle, making few movements during the day, except in response to changes in light. Consequently it was a comparatively simple matter to make observations.) By gradually moving the mirror it was possible to keep the mass of larvae moving under the blue and violet, thus driving them out of a corner and across the glass side of the box, however slowly. This reaction could not be secured by means of the red and yellow. The observations were carried on until 3:30 p.m.

III. THE INDIVIDUAL LARVAE AND ADULTS

1. Introduction

That the positive reaction to light as described in the last section is not the usual reaction of the single larva, is evidenced by the fact that the individual invariably goes away from the source of light. It has already been intimated that the older larvae are more strongly negative to light than the younger individuals. It is quite generally known that the adult flies are positive to light, i.e., fly toward the source of light. Hence, it is evident that the sarcophagids form a group of organisms whose reactions show progressive change; viz. first, during the feeding period the larvae as an aggregation of individuals react positively to artificial light though as individuals they are apparently strongly negative; secondly, the migrated larvae (in the prepupal period) are uniformily negative; and, finally, though the larvae pupate as negative organisms they emerge in a positive state.

It is clear that a series of experiments is needed on a number of individuals throughout their life history, at definite intervals and at a given light intensity, in order to determine the degree of reactiveness for the various periods, or stages. Further, the range of intensity through which the individuals react should be considered and determined. It has been shown in the earthworm (Parker and Arkin, '01) that there is a localization of the light receptive function; it is also quite likely that this function is

not distributed equally over the entire body of the fly larva, indeed Loeb ('90) has demonstrated that the anterior portion of the larva is most sensitive. Consequently it is highly important to locate definitely if possible the seat of the receptive organs in the sarcophagids.

When the imago emerges from the pupa case it has well developed organs of sight; viz. a pair of prominent compound eyes and three ocelli situated dorsally between them. The compound eyes of insects are regarded as image-forming organs, while the ocelli are often only direction eyes, analogous to the eyes of planarians, for example. Further experimental evidence with regard to these matters might well be secured from the flies in question.

Finally, it will be recalled that Lucilia caesar is essentially a fly of the out-of-doors, while Calliphora vomitoria frequents houses as well. Is there any relation between this difference in habit and the intensity of light to which the adults react?

These questions will be dealt with in the following pages.

2. Methods

Eighteen adults of Lucilia caesar collected October 1, 1907 formed the nucleus for the winter's supply of this species. From these adults (Lot no. 1a) five sets of larvae were obtained, viz. Lots nos. 2a, 3, 4, 5, and 6, the eggs having been deposited October 9, October 12, October 20, October 29, and November 1 respectively. Lot no. 5 proved to be the only successful one and from this a continuous colony was established for winter use. A continuous colony of Calliphora vomitoria was easily established from eggs deposited indoors by one adult female. Usually from two to six lots of adults of the two species were kept on hand, so that eggs and larvae were obtainable throughout the winter. The room in which the colonies were housed was constantly heated. The flies were exposed to the sunshine whenever available, which was an important factor in securing eggs.

The experimental work was carried on in a large basement dark room of the Museum of Comparative Zoölogy. Light intensities were measured and calculated in the usual manner with the aid of a Lummer-Brodhun photometer.

A series of incandescent lamps ranging from 4 to 100 cp. on
a 110–volt circuit were available and used as specified in the fol-
lowing experiments, as was also a Nernst double filament lamp
on the same voltage. A single filament Nernst lamp on a voltage
of 220 was used for the light grader mentioned farther on. A
200-cp. arc light and a low intensity apparatus described and fig-
ured by Adams ('03, p. 30) were utilized to secure the intensities
at the higher and lower extremes respectively. For reference and
to illustrate its use the low intensity apparatus is again figured
(fig. 1). Thus it was possible at any time to procure a light of
very high intensity or of very low intensity. The following list
of diaphragms were used with the low intensity box.

TABLE 2

List of diaphragms used with the low-intensity apparatus, together with the result-
ant intensities secured through reflected light from a 7.2 cp. incandescent light.

DIAPHRAGM.	DIAMETER OF THE CIRCULAR APERTURE	AREA OF APERTURE	INTENSITY IN C. M. AT CENTRAL SQUARE OF GLASS STAGE.
	cm.	*sq. cm.*	
A.....................	8.9	62.212	0.56
B.....................	5.0	19.635	0.1764
C.....................	2.2	3.8013	0.0342
D.....................	1.0	0.7854	0.00705
E.....................	0.5	0.19635	0.00176
F.....................	0.3	0.07068	0.00063
G.....................	0.1	0.00785	0.00007
H.....................	none		none

The low intensity box B (fig. 1) was so constructed that when
closed, light from an outside source could not enter it. In order
to note the position of the larvae at the beginning and end of a
given period a system of squares (each 1 sq. cm.) was constructed
by means of fine white threads crossed from side to side on an
oblong frame (E, fig. 1.) The squares were lettered and numbered,
the lettering A to I being transverse to the light rays, and the num-
bering 1 to 11 being in the direction of the rays, with the highest
number nearest the light. The frame rested on small blocks,
one under each corner, to allow space for the creeping animals.

The white thread could be plainly seen on the black background. The individual larvae were placed in the central square $E6$ as gently as possible by overturning the small pastboard box in which each was kept. The intensity calculations were made for the center of this square $E6$. By noting the square occupied by the larva at the end of a given period (*e.g.* thirty seconds), its movement with reference to the light could be easily ascertained and recorded. As illustration, a case may be cited as follows. The larva was placed in the square $E6$ and the dark box was quickly closed; after thirty seconds by the stop watch the box was opened and at a
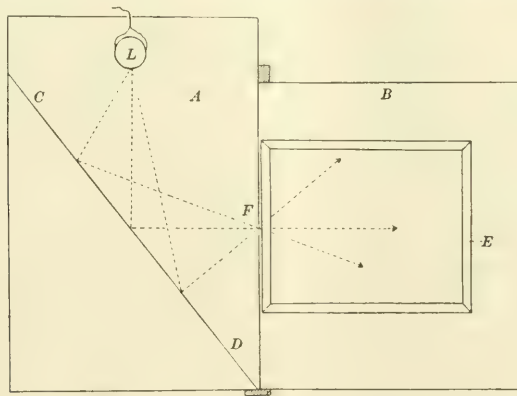
Fig. 1   Plan of low intensity apparatus. A, chamber containing 7.2 cp. incandescent lamp (L) and a vertical white screen (CD); B, a second dark chamber containing a glass stage upon which rests the reading frame E; F, diaphragm through which the light from L passes into chamber B after reflection from the screen CD.

glance it was seen that the larva was in square $G4$. This shows that the larva had travelled away from the light in a slightly diagonal direction. On the other hand, had the larva been found in $E9$ it is evident that a direction toward the light would have been taken.

Another piece of apparatus designated as a light grader by Mast ('06, pp. 363–366.) and also described in detail by him was constructed and applied with the results as set forth later in this paper. A single Nernst filament was used on a voltage of 220, which gave a light of 70.69 C.M. at the principal focus of the

cylindrical lens, *i.e.*, at the position of the centre of the stage. Using diaphragms with triangular openings and equal bases, but with different altitudes, light could be obtained which either graded off rapidly or gradually into darkness; but in case an ungraded field was desired, a diaphragm with a rectangular opening was substituted.

Fig. 2 illustrates in plan an apparatus which proved quite useful in producing a pencil of light both intense and well defined. A 77.4 cp. Nernst light was used at a distance of 50 cm., and the
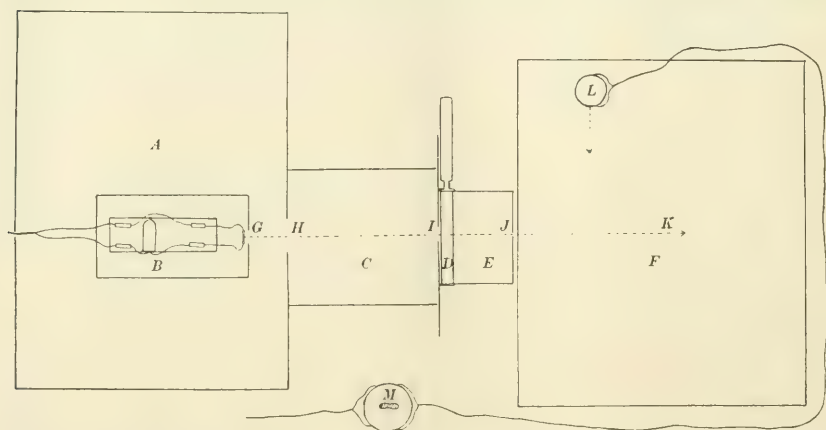
Fig. 2   Plan of light pencil apparatus.   *A*, chamber containing a Nernst lamp *B* (77.4 cp.); *G.* and *H*, diaphragms: *C*, a darkened cylinder with diaphragm *I; D*, reading glass; *E*, a second blackened cylinder with a pinhole diaphragm *J; K*, light-pencil; *L*, incandescent lamp used to start the larvae in the direction of the light-pencil *K*, and controlled by the switch *M; F*, a sheet of smooth slate used as a stage for the larvae to creep upon.

light pencil was from 1 mm. to 2 mm. in width at the point of experimentation, varying with the path of the traveling larva. There was so little diffusion that it was quite impossible to see the moving individual until it came into the light at right angles to its own path.   In order to start the larva in the proper direction, the light *L* (fig. 2). controlled by the switch *M* was turned on for a few moments and again turned off as soon as the larva began moving in the desired direction.   By using a smaller stage and suspending it, the light pencil could be played on the larva at will.

To secure tracings of the paths of larvae, several methods were tried, of which the most satisfactory proved to be that of starting the larva off from a drop of very weak aqueous solution ($\frac{1}{8}$ to $\frac{1}{4}$ per cent) of methylene blue, whereby a trail was made on paper by the creeping larva. By comparison with trails made with the use of tap water, it could be seen, as might have been expected from the weakness of the solution, that the methylene blue had no disturbing effect. The larvae were started in a drop of the fluid enough adhering to them to leave a distinct track on the white paper, portraying nearly every movement made, except when the head was raised high. White paper did not give results different from those obtained with black paper, except when used in the light grader with light from above. In that case reflection was eliminated by means of a diaphragm, which was removed when the larva entered upon the edge of the field.

3. Reactiveness and age

Pouchet ('72, p. 312) suggests that the larva avoid the light immediately upon hatching, but that they are not able to react to its direction. This function develops progressively as the individual grows older and does not attain full force until the larva has completed its growth. This seemed to be an important matter to decide definitely, since all conclusions must in the end be based on the reactiveness of the individual at a given period in its life history.

To test this it was decided to use the low-intensity apparatus with an intensity of 0.56 C.M. In this way reflection can be largely eliminated, and by means of a low intensity of light are avoided such matters as heat and overstimulation both of which produce an "excited" state of the organism. The feeding larvae were permitted to crawl on a glass plate well smeared with fish and remoistened frequently in order to guard against the possibility that one individual might follow the trail of another. By examination of the data it was evident that successive trails were seldom alike, and my observations satisfied all doubts in the matter. The larvae must either be moist themselves or creep on a moist sur-

face, otherw'se they roll over and over in attempting to creep on a smooth, dry surface. The plate smeared with fish juices prevented the unequal distribution of odors which might complicate the movements of the feeding larvae. The migrated larvae on the other hand were allowed to creep on glass which was frequently sponged with tap water to provide a moist surface on which the larvae could crawl and to obliterate previous trails.

The data for table 3 are based on a series of experiments on the ten individuals throughout their life history, except when in a few cases new larvae were substituted for those accidentally lost. Each was isolated after hatching by being placed in a small cardboard box with its own piece of fish. The results of this series of experiments are tabulated as follows:

TABLE 3

Summary of reactions of Lucilia caesar (Lot no. 10) at different ages to directive light (0.56 C.M.), based on ten larvae each given five trials with an exposure of thirty seconds.

DATE	AGE	STAGE	REACTION					
			Trials			Percentage		
			+	−	0	+	−	0
1907								
Dec. 5, 10.45 a.m.	Birth {	Just	a, 6	5	7	a, 40	25	35
		hatched	b, 1	11	8	b, 5	55	40
Dec. 6, 10.45 a.m.	24 hrs.	Feeding	6	31	13	12	62	26
Dec. 7, 10.35 a.m.	48 hrs.	Feeding	3	36	11	6	72	22
Dec. 8, 10.35 a.m.	72 hrs.	Feeding	1	42	7	2	84	14
Dec. 9, 10.45 a.m.	96 hrs.	Feeding	0	44	6	0	88	12
Dec. 10, 10.35 a.m.	110 hrs.	Feeding	0	47	3	0	94	6
Dec. 11, 10.35 a.m.	6 ds.	Prepupal	1	47	2	2	94	4
Dec. 12, 10.40 a.m.	7 ds.	Prepupal	2	48	0	4	96	0
Dec. 13, 10.35 a.m.	8 ds.	Prepupal	0	50	0	0	100	0
Dec. 14, 10.30 a.m.	9 ds.	Prepupal	3	46	1	*6	92	2
Dec. 16, 10.30 a.m.	11 ds.	Prepupal	2	28	0	*7	93	0
Dec. 17, 10.30 a.m.	12 ds.	Prepupal	0	20	0	0	100	0
Dec. 18, 10.30 a.m.	13 ds.	Prepupal	0	10	0	0	100	0
Dec. 26–30	25 ds.	Imago	27	†1	0	97	3	0

* See table 4 for same date.

† This aberrant reaction was on the part of an individual whose wings did not spread; it was consequently forced to creep. All other adults first perched on the edge of the vial in which they were retained and then flew toward the light.

Table 3 shows clearly that the larvae grow more and more sensitive to directive light and that they are perfectly negative when the feeding period has ended.

The newly hatched larvae appeared to be indifferent to the direction of the rays, but an exposure of three minutes to a high intensity (336 C.M.) showed quite conclusively that even these young larvae were negatively influenced by directive light.

The imagoes emerge in a positive state. The small percentage of negative reactions is based on a single reaction of one individual whose wings were not spread, hence this behavior should be regarded as accidental.

A record of the individual larvae is given in table 4 and is serviceable in locating irregularities, *e.g.*, the readings of December 14 and 16. These aberrant reactions are based on one individual (no. 8), which proved to be very "excitable" under mechanical stimulation; thus, a slight pressure or dropping carelessly caused the larva to start moving at once without first orienting itself to the light. This table also shows that there is no di er-ence between the reactions of the males and of the females to light.

4. *Reactiveness and intensity*

Even the casual observer must be familiar with the general negative reaction of the migrated fly-larvae, observable through a considerable range of intensities, *i.e.*, to bright sunshine, to diffuse daylight, and to ordinary lamplight. It has already been pointed out that the migrated larvae of Lucilia caesar are uniformly negative to directive incandescent lamplight of 0.56 C.M. intensity. It remains to be seen what the degree of sensitiveness is at lower intensities. Since the reaction of the migrated larvae is quite uniform, the series of experiments with reference to sensitiveness was made during the prepupal period. The methods described in section 3 for larvae at this period were likewise applied in this case, as was also the low-intensity box with the diaphragms listed in table 2. The individual was placed in Square $E6$ (the central square of the reading frame, fig. 1, E), the dark chamber (B) was closed, and after the lapse of thirty seconds the posi-

TABLE 4

Summary of individual reactions of Lucilia caesar (Lot. no. 10) to directive light (0.56 C. M.).

NUMBER OF THE INDIVIDUAL	1			2			3			4			5			6			7			8			9			10		
Direction of the reaction.	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0	+	−	0
Dec. 6, 1907 10.45 a.m.	2	2	1	0	3	2	1	3	1	0	4	1	0	4	1	1	3	1	2	3	0	0	1	4	0	4	1	0	4	1
Dec. 7, 1907 10.35 a.m.	0	3	2	0	1	4	0	4	1	0	4	1	0	5	0	0	5	0	1	2	2	0	5	0	1	4	0	1	3	1
Dec. 8, 1907 10.35 a.m.	0	4	1	0	4	1	0	5	0	1	4	0	0	5	0	0	4	1	0	4	1	0	5	0	0	3	2	0	4	0
Dec. 9, 1907 10.45 a.m.	0	3	2	0	4	1	0	5	0	0	5	0	0	4	0	0	5	0	0	5	0	0	5	0	0	4	1	0	4	1
Dec. 10, 1907 10.35 a.m.	*0	5	0	0	5	0	0	4	1	0	5	0	0	5	1	*0	5	1	0	3	2	0	5	0	0	5	0	0	5	0
Dec. 11, 1907 10.35 a.m.	M0	5	0	M0	5	0	M0	5	0	M0	4	1	M0	5	0	M1	3	0	M0	5	0	M0	5	0	M0	5	0	M0	5	0
Dec. 12, 1907 10.40 a.m.	0	5	0	0	5	0	1	4	0	1	4	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0
Dec. 13, 1907 10.35 a.m.	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0
Dec. 14, 1907 10.30 a.m.	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	†3	2	0	0	4	1	0	5	0
Dec. 16, 1907 10.30 a.m.	0	5	0	0	5	0	P. 15th.			0	5	0	P. 16th			0	5	0	P.15th			2	3	0	P. 16th			0	5	0
Dec. 17, 1907 10.30 a.m.	0	5	0	P. 17 th.						0	5	0				P. 17 th						P. 16 th						P. 15 th		
Dec. 18, 1907 10.30 a.m.	0	5	0							‡ out																				
Dec. 19, 1907	P. 19th																													
Dec. 26, 1907	♂5	0	0	♂ Dead			♂ Too weak						♂5	0	0	♂5	0	0	♀5	0	0	♀5	0	0	♂4	1	0	♀ Dead.		
Dec. 30, 1907	0	0																												

*New individual introduced.

M = Migration; P = Pupation.

†Because of the unexpected behavior of no. 8 on this date, the check lot of 55 individuals was tried, only three giving aberrant reactions, all others negative. These three were isolated and their daily records taken for several days, resulting in a total of 9% +, 85% −, and 6% 0, including the first aberrant reaction of each. It was also found that these individuals were extraordinarily sensitive to mechanical stimulation, which accounts for the results obtained.

‡This individual did not pupate until March, hence it was taken out of the series.

tion of the larva was noted and recorded.   Records in total darkness were taken in the same manner with the aperture for light entirely closed.

Table 5 is a summary of all records for both species.

TABLE 5

*Reactiveness to directive light through the general range of intensity for migrated larvae of Lucilia caesar   (Lots no. 6 [A] and no. 25 [B]), and of Calliphora vomitoria (Lot no. 24.)**

		REACTIONS					
SOURCE OF LIGHT	INTENSITY IN C. M.	L. caesar			C. vomitoria		
		+	−	0	+	−	0
Diffuse daylight.......	?	(A) 0	50	0	0	50	0
Arc light..............	800.	(A) 0	50	0	0	47	3
Incand. light..........	0.56	(A) 1	44	5	3	44	3
Incand. light..........	0.1764	(A) 5	37	8	8	32	10
Incand. light..........	0.0342	(A) 6	35	8	2	35	13
Incand. light..........	0.00705	(B) 0	33	18	7	24	19
Incand. light..........	0.00176	(B) 2	28	20	8	17	25
Incand. light..........	0.00063	(B) 5	14	28	9	11	30
Incand. light..........	0.00007	(A) 1	3	46	5	4	41
	Total darkness	(A) 2	2	46	5	1	44

* The reactions (+ − 0 = positive, negative, and indifferent respectively) are based on the movements of ten larvae given five trials each with an exposure of thirty seconds. Between trials—taking larva no. 1 first, then no. 2, etc., through the series—each individual was kept separate in a closed receptacle.

An inspection of table 5 shows that in Lucilia caesar the lowest directive intensity is 0.00176 C.M. and for Calliphora vomitoria, 0.00705 C.M. This difference in sensitiveness was quite evident in all experiments in which both species were involved, L. caesar responding more readily and being more active than C. vomitoria.

Below the respective minimum directive intensities, light has, however, a dynamic effect until an intensity of 0.00007 C.M. is reached, when there is neither a directive nor a dynamic effect. The larva under such conditions remained perfectly quiet, as though in the dark. This is illustrated by fig. 24, which shows the tracings made by larvae in total darkness. There is a certain

amount of stimulation due to rolling the larva into place; under such conditions it makes many randon movements, is clearly undirected, and soon comes to rest.

The large number of indifferent reactions recorded for the lowest intensity and total darkness is based on the behavior just described; whereas the reactions recorded in the same column for higher intensities may also be based on movement to right and left, neither positive nor negative (commonly designated as zero movements).

5. Light graded in intensity

Two methods of light grading were employed. The first was by means of the light grader and was of non-directive nature i.e., vertical light directed on a horizontal stage on which the animal moved. A field of light was thus secured ranging from a maximum of 70.69 C.M. to 0 within a given distance. The second method, illustrated by fig. 11, gave a field of graded light directive in its nature, but gradually increasing in intensity over a given area in the direction of the rays. Such a field was produced from the vertical filaments of a 66 cp. incandescent lamp (L) by placing an opaque screen (C) in front of it in such a manner that the light from the entire length of the filament could reach the horizontal stage at a point (B), one metre distant from the uppermost portion of the filament. On both sides of this point (B) the light gradually diminished in intensity; toward the filament it diminished because less and less of the filament was in range of the stage, and away from the filaments it diminished because of the radiation of the light.

The behavior of the larvae in reference to the two methods will be discussed separately: first, behavior under non-directive graded light, and secondly, behavior toward directive graded light in the above sense.

A. *Non-directive graded light.* A field of light of this character was produced by means of the light grader already mentioned. Triangular diaphragms of various altitudes were used, the extreme lengths producing fields of light 2 cm. broad and respectively 10 cm. and 2 cm. long. The intensity of the light in the strongest

region was 70.69 C.M. and it gradually diminished to nothing at the other extreme.   Of the two extreme lengths mentioned, the longer resulted in a gradation of 7 C.M. per centimetre and the shorter in a gradation of 35 C.M. per centimetre.

Since the sarcophagid fly-larvae are negative to light, one would expect them to turn to the darker portion on entering such a field of graded light.   This would also be expected when one considers the results obtained by means of light on two sides, as illustrated by figs. 3 and 4.   The courses of the larvae always lay more or less transverse to the rays, the greater deflection being toward the light of lower intensity regardless of the size of the luminous field.   When the larvae were placed midway between two balanced lights of like luminous area (likewise when of unlike area, but like intensity), their course lay transversely to the rays, as illustrated by fig. 5.   These results are in accord with the statements made by Loeb ('05): "If there are two sources of light of different intensities, the animal is oriented by the stronger of the two lights. If their intensities be equal, the animal is oriented in such a way as to have symmetrical points of its body struck by the rays at the same angle."

If, now, the negative fly larva is in a field of graded light, one would expect it in creeping to take a course toward the darker side of the field until stimulation (light from above)became equal on both sides, and decreasing in intensity.   Clearly the opposite direction would be out of the question, since that involves a gradual increase in intensity, though there would be a chance for equal bilateral stimulation.   Since locomotion is involved and consequently directive stimulation in order to bring the larva in right relation to the field of light, it was necessary to start the animal off in the proper direction by means of a light from behind the larva. which could be controlled.   This was first accomplished by means of a 7 cp. incandescent light.   Thus the larva was properly oriented and would continue traveling in the same direction for some time after the light was turned off.   The more frequent result on reaching the graded field was that the larva passed into this area of light, exhibiting the usual random movements, but the *after effects* of the directive light, though the light was turned off ten

to twenty seconds before, were sufficient to keep the larva moving in the general direction taken at first (fig. 7a). Less frequently the larva withdrew after a few random movements and then took a course along the border of the field either to the right or to the left (figs. 6 right hand tracing and 7b), or in a very few cases turned directly back on its own course.

Apparently the "starting off" was brought about by means of an intensity too high in proportion to the non-directive light. Therefore in all later experiments a directive light of about 0.5 C.M. was employed. Under such conditions the larvae very seldom went into the field of light: but, once in the field, they passed through in the manner described, again with no constant relation to intensity. It was then decided not to turn off the directive light, but keep it on the larva continuously. The result of these experiments is shown in fig. 8, which represents the usual reaction either to right or left. The larva on arriving at the edge of the field of higher intensity withdrew and then moved somewhat transversely to the directive rays which however, soon caused it to take up its usual longitudinal orientation, as a result of which if once more entered the more intense field, but again withdrew. This behavior continued until the region of higher intensity was passed whereupon the course finally followed the rays of the directive light.

Over 150 recorded experiments, as illustrated by the figures were made for fields varying from 10 cm. to 2 cm. in length. An equal number of unrecorded experiments (*i.e.* unrecorded by larval traces) were made in which the light passing through the plate glass stage was reflected out of the light grader by means of the mirror always in place. There is clearly no constant relation between the movements of the animals and the graded field.

It was further found that the larvae could be forced entirely through the graded field from the dark (0) end to the brightly illuminated end (70.69 C.M.) when an intensity of 50 C.M. or over was used to direct them. This was not possible when the directive light had an intensity of only 0.5 C.M. Under this intensity the larvae would, however, pass farther into the darker end of a field grading at the rate of 7 C.M. per centimetre, than into one grading at the rate of 35 C.M. per centimetre. Though

the relation was not constant the usual distance was about five times as far in the former field (fig. 9 and 10).

Mast ('07) found that Volvox, which is positively phototatic, was deflected toward the more strongly illuminated side in graded light and concludes (p. 141) that "The direction of motion in Volvox exposed to light is consequently regulated by the intensity of the light on opposite sides of the colonies regardless of the direction of the ray." This conclusion, as Mast shows, is in direct opposition to the statement made by Loeb ('05) and already referred to.

From the experiments already enumerated and others to follow (B), it becomes quite evident that the results obtained with sarcophagid fly-larvae are quite in accord with Loeb's ('05) conclusions, and that the statement made by Mast ('07, p. 136) is quite apropos at this juncture. "Let it be clearly understood that in the criticism of Loeb's conclusions, I do not wish to intimate, that because the reactions of Volvox or any other organism do not take place in accord with those conclusions, they necessarily cannot hold for the organisms Loeb worked with."[5] Mast's further statement on the same page in reference to that investigator's results is, however, equally applicable, viz. "I do, however, wish to state and emphasize that in my opinion his experimental results as quoted above, do not warrant his conclusions, even for the animals worked on, much less for all organisms which orient to light."

B.   *Directive graded light.*   A field of light, directive with reference to the larvae, graded to higher intensities in the direction of the ray was produced in the manner already explained. The field (AB, fig. 11) was 36 cm. in length and 25 cm. in width, providing ample room for long journeys under these conditions. The grading was from the point A, with an intensity of less than 1 C.M., over a distance of 36 cm. to a point B, with an intensity of 66 C.M. The larvae were started about 2 cm. beyond the point A, where orientation away from the light took place. Locomo-

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[5] Loeb's conclusions, were based on results obtained from experiments on blow-fly larvae among other species (Loeb, '90, pp. 70–71).

tion under such circumstances brought the larvae into successively higher intensities, and in consequence numerous trial movements were produced and creeping ahead was accomplished very slowly. As the larva advanced into regions of still higher intensities, its trial movements were continued, though reduced in number, but the rate of locomotion was increased. A very pronounced change in behavior was evident when the point B was passed and the path lay in light decreasing in intensity. The rate of movement
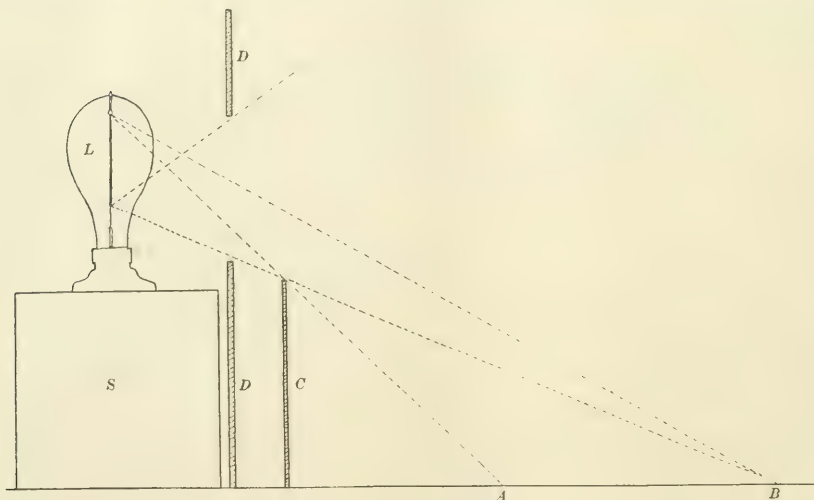
Fig. 11 Outline of apparatus to produce a field of light increasing in intensity in the direction of the rays. L, a 66 cp. incandescent lamp elevated on a stand (S); C, an opaque screen; DD, an opaque screen with an oblong aperture about the size of the incandescent bulb, to eliminate side light; AB, a field of light produced by the lamp (L), gradually increasing in intensity from A to B.

increased perceptibly, trial movements were visibly eliminated, and the course was more nearly a straight line. The rate of movement for a distance of 10 cm. on the brighter side of the point B was greater by 10 per cent than for a like distance on the other side of this point. This average is based on continuous movements of ten larvae each given a single trial after 12 to 14 hours of rest.

In the field AB there was a very marked tendency on the part of the larvae to take a diagonal course to the right or the left. Out of 50 trials on 10 larvae, 50 per cent of the paths were diagonal,

whereas under usual conditions with like intensity such diagonal courses were reduced to about 15 per cent in the same number of trials. Out of 50 trials, 4 per cent (two trials by the same larva) resulted in a return to the point A, the larva refusing to go into regions of higher intensities. This larva in its remaining trials (three in number) took a sharply diagonal course.

Under the heading of "Accuracy of Orientation," Walter ('07, pp. 79–80) notes that the negative planarians subjected to directive light showed a strong tendency to take a path in a diagonal direction, and calls attention to the similar case found by Smith ('02, p. 469) for the earthworm. Walter believes this to be due in the case of planarians, to imperfect orientation resulting from the crescentic pigment shields of the eye, which would permit a diagonal path to the right or left to a certain degree without allowing light to stimulate the retina. In the eyeless earthworm, though Walter does not suggest this, the diagonal path may have been due also to imperfect orientation. Furthermore, the arrangement of the sense organs on the segments, as shown by Harper ('05), would certainly permit a more or less diagonal course, $i.e.$, a turning from side to side to a certain degree as the worm crawls would be possible without subjecting the sense organs to stimulation from light.

A very much more perfect orientation is possible on the part of the fly-larva because of the localized condition of the photoreceptive function as discussed on page 205, etc. In these organisms the receptive surface is restricted to the extreme anterior pole, and as the animal travels away from the light, this part of its body lies in its own shadow, so that the creature is continuously oriented within a narrow range of shadow.

6. Intensity and rate of movement

While experimenting with various intensities of light, it became apparent that the larvae crawled more rapidly as the directive light became more intense. The question naturally arose,—What is the relation between the intensity of the light and the rate of movement. This matter was tested in the following manner.

The larvae were started in directive light from a drop of tap water (slightly colored with Methylene blue), which afforded the necessary moisture for crawling upon a sheet of paper. The individual was given two to three centimetres in which to gain proper orientation. This brought its extreme posterior end on the starting line, from which the larva was timed by means of a stop-watch over a distance of 10 cm. The course of these animals when once properly oriented is nearly straight, and the time is based on continuous movement; larvae which paused in the course were removed and not used again till after they had rested. This measure was very seldom necessary. The light intensity was calculated for the middle point of the course, since the results in rate will be more nearly in accord with the given intensity as an approximate average.

The tabulated results are based on the movements of ten larvae, each given a single trial (except as above mentioned), with a rest of from one half to one hour before exposure to the next intensity. This method was pursued in order to guard against excessive mechanical stimulation or exhaustion. No heat screen was used, since no difference was found in average between the rates of larvae in high intensities with and without a receptacle of water interposed to absorb the heat.

TABLE 6

The relation between the intensity of directive light and the rate of movement of sarcophagid larvae, based on the time in seconds required for migrated individuals to travel ten centimetres. The intensity of the light is calculated for the middle point in the course.

SOURCE OF LIGHT	INTENSITY IN C. M.	RATE FOR 10 cm.	
		L. caesar	C. vomitoria
		sec.	sec.
Incandescent...........	0.00176	30.10	
Incandescent...........	0.00705	29.66	
Incandescent...........	0.56	27.64	55.86
Incandescent...........	325.0	24.02	50.28
Incandescent...........	1057.0	21.34	37.70
Arc light...............	5000.0	18.86	29.16

One must conclude from the summary of the experiments in table 4 that the rate of movement increases with the intensity. Davenport and Cannon ('97, p. 32) say for Daphnia, "Since there is no close relation between diminished intensity and the longer time required for migration, it seems more probable that this longer time is not the result of lower intensity, but that it is due to diminished precision of orientation showing itself in hesitating movements." Although this statement was made for a positive organism, it might nevertheless be inferred that the same conclusions would hold for negative organisms as well. This is in part true of the sarcophagid fly-larvae. Certainly there are many more random movements in very low intensities, as illustrated by fig. 16 (p. 219); these movements disappear largely if not entirely at high intensities, e.g. fig. 19 (p. 219). But it cannot be concluded that the increase in rate of movement in high intensities is due entirely to precision of orientation, since the light not only has a directive but also a dynamic effect. In table 5 it is shown that there is such a result even when the directive element has ceased. By closely observing the larva as it travels in high intensities, e.g., 1000 C.M., it can readily be seen that the crawling movements are greatly accelerated. This was particularly noticeable when the larva was under the conditions shown in fig. 4. When the animal passed into the field of higher intensity (1056 C.M.) its longitudinal contractions and elongations were perceptibly increased in rapidity after re-orientation, and the sidewise movements of the head were visibly less.

Again, in a series of experiments discussed on page 199, in which the larvae were moving away from the source of light in a field gradually increasing in intensity, both phenomena were evident, viz., the sidewise movements decreased in number, while the rate increased. The sidewise or random movements were, however, greatly exaggerated under the conditions already pointed out.

Yerkes ('00) in a further study of Daphnia also concludes with Davenport and Cannon that the increase in rate for these forms depends chiefly upon precision, but found evidence of a "quickening of the swimming movements."

Davenport and Cannon found that the relation existing between the rate of movement (Daphnia) and the intensity of the light could be expressed thus: with one-fourth light, about 118 per cent of the time with full light. Yerkes expressed this in the following ratios: ratio of intensities 5.12: 1, and ratio of rates 1:1.25. By calculation such ratios may be roughly approximated in table 6, however, only with regard to the higher intensities. No like ratios could be established for the light of lower intensities, since under such conditions the course of the larvae is not continuous, but is greatly influenced by random movements. It must also be noted that the maximum rate for any single larva (1.64 second per cm.) was already attained in the 1057 C.M. intensity by one individual and not exceeded in 5000 C. M., though the average for this intensity is 1.88 sec. per cm. (See also Nutting, '08, in this connection.)

It is apparent that the rates for the two species are quite different, but the ratios hold approximately for each. It was quite out of the question to secure a set of even approximately uniform rates in low intensities for C. vomitoria, because of the extreme individual variation, due to hesitation and wandering.

7. Sudden change in intensity

One is naturally led to ask the question: What is the natur⁁ of the response when a light is thrown suddenly from above upon the animal as it creeps in the dark or in an illumination of low intensity. If one considers for a moment what the result would be upon an animal having eyes, one might be led to expect a similar result under similar conditions when a fly-larvae is suddenly illuminated, viz. that it would pause momentarily, at least, and probably be thrown out of orientation. But it must be considered that in the sarcophagid larva we have to deal with an eyeless organism. Various observers have found that certain organisms are thus affected to a greater or less degree. Yerkes ('00, pp. 416–417) found that neither Daphnia nor Cypris responded by turning, but that both species quickened their movements as the result of sudden illumination from above. Jennings ('04,

pp. 49–50) describes what he calls a typical motor reaction for Euglena when, swimming toward the source of light, the illumination is suddenly decreased. Mast ('06, p. 370) found that "Stentors which are oriented to a given light respond with the motor reaction to an increase in intensity of the light, they are for the time being thrown out of orientation." It was also found by Walter ('07, p. 63) that Planaria gonocephala "showed a decided response —either some change in course or a wigwag motion of the anterior end—more frequently when suddenly subjected to dark than to light."

To test the larvae of the sarcophagids to sudden changes of intensity, the light grader was employed so as to throw light from above upon the animals as they crawled on the glass stage at the focus of the lens. By means of a rectangular opening, instead of one triangular in outline, an ungraded field of 70 C.M. throughout was produced. The larvae were caused to crawl in the direction of a given area which could be suddenly illuminated by withdrawal of a diaphragm, when the individual had reached the proper position.

The results were quite uniform; whether the larvae were crawling in the dark, in 0.5 C.M., or in 50 C.M. directive light, all produced random movements, and were more or less thrown out of orientation. The *after-effects* of the directive light on the larvae traveling in the dark, were sufficient to cause such individuals to retain the original general direction even after having been suddenly illuminated. The accompanying figure (fig. 12) shows clearly the nature of the reaction. On the other hand by a comparison of fig. 16 to 19 it is quite evident that sudden decrease in intensity, *e.g.*, decrease from an intensity of 960 C.M. to total darkness, does not throw the larvae out of orientation, nor does it cause any apparent disturbance at the moment.

8. *Localization of the function*

It has already been pointed out by Loeb ('90, pp. 71–72) that only the rays of light striking the oral pole of the larva are effective in orientation. This conclusion, however, was reached by means

of rather crude methods: the larvae were placed on a board, which was thrust forward out of the shade in such a manner that the oral pole of the larva was subjected to sunlight. This caused the animal to withdraw its head and take up a position parallel with the rays. A similar experiment with reference to the aboral pole of the larva did not result in a like orientation. Severance of the anterior segments also resulted in an inability to orient when subjected to light. Loeb rightly lays little stress on the results of this latter method.

The experiments cited are characterized by the following statement made by Loeb earlier in the same paper (p. 21). "Die Thatsachen die ich nachzuweisen habe, sind von so einfacher Art, dass fast jedes technische Hülfsmittel dabei entbehrt werden kann." Later observations by many different investigators have proved that the reactions of the lower organisms to light are not of the simple nature inferred by Loeb.

It will be observed that the position often occupied by the feeding larvae in reference to light must lead one to suspect that the posterior parts are not strongly sensitive, if at all. The head under such conditions (*i.e.*, feeding) is buried in the tissues of the flesh, while all the rest of the body may be protruded in full daylight. Furthermore, when the larva travels away from a source of light, its aboral portions are fully exposed to the light, while the head is obviously kept in shadow as much as possible. The manner in which the larvae wave the head about in response to weak intensities leads one also to suspect that the rays falling on this part serve as a directive agency.

A pencil of light may be employed with which to explore the entire body of the larva in order to ascertain the sensitive regions or region. Even with a fine pencil of light there will be some diffusion, but by means of a pinhole aperture (fig. 2, *J*) diffusion can be reduced to a minimum as compared with the length of the larva.

All parts of the length of the animal were carefully explored, but only one region was found where the light caused the larva to turn, and that was at the very tip of the oral end. By throwing the pencil on this region continuously, the larva could be forced to crawl in a circle as illustrated by fig. 13. Blackening this region

with a mixture of lard and lampblack, and then illuminating that portion, failed to produce any turning until the substance was rubbed off in crawling. On blackening one side of this region only and exposing the larva to light from overhead there were produced the typical circus movements with the pigmented side toward the centre of the circle, as found by Holmes ('01) for negative terrestrial amphipods. It should be said that each larva was first tested for the normal reaction before the pigment was applied.

It was also possible by careful manipulation to snip off the segment possessing the anterior hooks (the first segment), but the results were most unsatisfactory, since the larva is almost wholly dependent upon these hooks for locomotion, and consequently its reactions were questionable. Moreover, larvae thus operated on die in a few days.

Another series of experiments was tried with the light pencil apparatus, which gave further evidence toward the restriction of photo-sensitiveness to a very limited region at the oral pole of the larva. The individuals were started from a drop of tapwater on the slate stage (fig. 2, F) toward the light pencil (K). As soon as a fair start was made the light (L) was turned off. If the conclusion already reached is to hold good the fly larva should be more or less sharply deflected to its left side on encountering the pencil of light. The moisture on the animal from the drop of water left a trail on the black stage so that its movements in the dark could be traced after again turning on the light.

Ten migrated larvae of C. vomitoria were tried, each five times, and out of this total of fifty trials with the light striking the right side of the individual 80 per cent of the courses were deflected to the left. When the light pencil acted on the left side of the larva, again 80 per cent of the courses were deflected, but this time to the right. It is quite evident that this signifies a well balanced bilateral condition of the sense organs.

The remaining 20 per cent of the trials represent courses taken straight through the light pencil without deflection; there were *no* deflections *toward* the source of light. If any deflection was to take place, it occurred each time when the head of the larva came into the light; as soon as the head passed through the illuminated

area into the darkness beyond, no further deflection took place. The light pencil at the point of experimentation was about 2 mm. in width, and individuals which had gained unusual headway might rush, so to speak, through the light because of its narrow proportions. The larvae usually paused on encountering the light and made trial movements in a very striking manner, occasionally
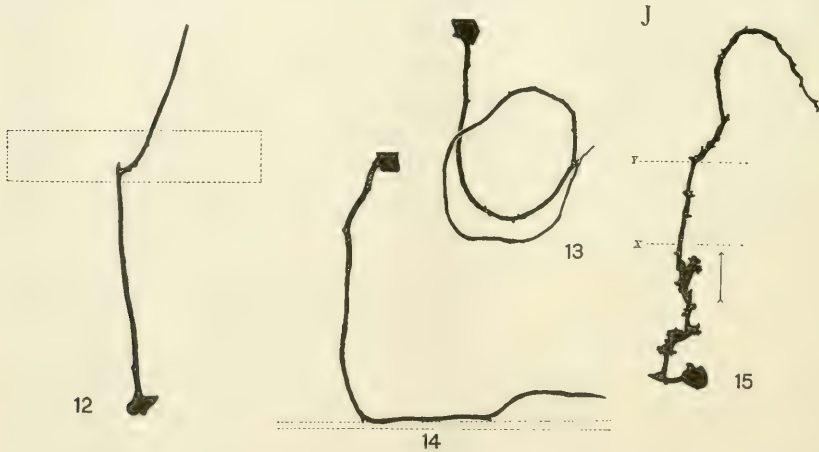
Fig. 12 Lucilia caesar.   Course of a larva that was creeping in a directive intensity of about 0.5 C. M., when ungraded light of 70 C. M. was thrown upon it from above. Orientation is clearly influenced.

Fig. 13 Course of a larva of Lucilia caesar when the light pencil is played continuously on its head.

Fig. 14 Lucilia caesar. Typical course of the larvae when encountering the pencil of light.

Fig. 15 Course of a larva of C. vomitoria showing the influence of the after-effects of directive light. The individual was started under a directive intensity of 3 C. M. and crept in total darkness from X to Y, when a new directive intensity of 0.56 C. M. was applied from in front.

stretching the anterior segments so far that the darkness on the other side was reached, when the larva continued on its way.

The larvae of Lucilia caesar were not given as many trials, but ten larvae, each given one trial for the right and one for the left side, were deflected sharply each time as is illustrated by fig. 14. This again bears out the conclusions that this species is more sensitive to light than C. vomitoria.

9. *The adult sarcophagid*

In an earlier paper on the sarcophagids, I ('07, p. 49) assumed that the eyes of these animals are of much importance in orientation, because of their relatively large size. But attention was also called to the fact that the chemical sense is probably of more importance in detecting the presence of food, since it could hardly be assumed that the vision of these animals is so acute that so small an object as a fish could be seen at any great distance. On several occasions a dead fish wrapped tightly in folds of paper was carefully enclosed in a tight box so that odors from the fish could not be detected. The package was then carried to a location far removed from the beach where no flies were to be seen and there opened up and the fish exposed. In ten minutes many sarcophagid flies were hovering about, and some eggs had already been deposited. It can hardly be assumed that these flies found the fish through sharpness of vision.

It is the object of the following series of experiments to test the eyes of these flies for their image forming powers. Other insects have been worked on to ascertain their powers in this respect, among them the mourning cloak butterfly (Vanessa antiopa) by Parker ('03) and Cole ('07), which species will form a basis for comparison.

A second question to be considered is one of distribution, alluded to in the Introduction, viz: Is there any relation between the phototropism of the two species and the fact that L. caesar is primarily a fly of the fields and C. vomitoria more or less a household form.

An answer to both of these questions may be sought by the use of an apparatus described by Cole ('07, pp. 340–346), but somewhat modified to meet the present need.

The method and apparatus may be briefly described as follows. Two lights of different areas were used, one, a single Nernst filament on a 110-volt circuit placed back of a metal sheet in which a narrow slit was cut; the size of this slit could be regulated by means of a sliding shutter. The second light was produced by reflection upon a vertical plate of ground glass. The source of this reflected light was a two-filament Nernst lamp whose rays were thrown upon

a vertical white screen standing at an angle of 45° to the ground glass. Placing a photometer midway between the two areas the lights were balanced by manipulating the sliding shutter. The area of the smaller light after balancing was 7 sq. mm., while the area of the larger field (the illuminated ground glass) was 52,800 sq. mm., or a ratio of approximately 1:7500. The intensity of each source was 12 cp.

The first set of experiments was tried on the migrated larvae. The individuals were placed midway between the two lights, and in all cases they took a straight path without turning toward either light. This behavior would be expected under light of equal intensities and at equal distances acting bilaterally upon these organisms. It must therefore be concluded that the size of the luminous area has no influence on the movements of the larvae.

To test the adults a glass cylinder 20 cm. in diameter and 25 cm. in height was placed between the two lights so that the axis of the cylinder coincided with the region of equal light intensity. A small glass vial with a rectangular stage of black cardboard around its neck served as receptacle for the individual flies as they were tried. After transferring the fly from its original receptacle into the vial, the latter was placed inside the glass cylinder, tilted so as to rest on the edge of the stage with the longitudinal axis of the vial on the line of equal light intensity. The cylinder was then covered with a sheet of black cardboard. The flies, which are negatively geotropic, naturally crawled up the vial and emerged with the light from either side striking the two eyes equally. Under these conditions fairly accurate results might be expected. On protruding the head from the mouth of the vial the fly usually paused for a moment, then either crawled out upon the stage or flew immediately to one side or the other of the cylinder. Less often it crawled the entire distance. In transferring the individuals from one vial to another their negative geotropism and positive phototropism were made serviceable. A little practice and precaution were necessary to recapture the individuals after liberation in the cylinder without crushing or losing them.

Ten specimens of each species were exposed first to the two areas separately and then to the two simultaneously.

By inspection of table 7 it may be seen that Lucilia caesar is much more strongly phototactic than Calliphora vomitoria, for under all conditions the former turned more frequently toward the single light or the larger area than the latter.   It should also be added that Lucilia caesar is far more reponsive to light, as is evident from the following data.   The average time that expired between the moment when the head of the fly was protruded from the mouth of the vial and the time of arrival at the side of the cylinder, based on 25 reactions of ten individuals to the larger area alone, was 8.1 seconds for L. caesar and 21.8 seconds for C. vomitoria.

TABLE 7

Summary of reactions of adult sarcophagids to opposing lights of the same intensity, but form sources whose areas were as 1 to 7500.

	C. VOMITORIA				L. CAESAR			
	Response in percent			Number of trials	Response in percent			Number of trials
Direction of reaction..	+	−	0		+	−	0	
To area 1 (alone)........	62	22	16	50	100	0	0	25
To area 7500 (alone)...	72	18	10	50	92	8	0	25
To area 7500 (both used simultaneously)	62	36	2	100	69	28	3	76

In view of these facts an answer may be given to the second question proposed at the beginning of this section. The more frequent presence of C. vomitoria in houses and like situations is due chiefly to its relatively low degree of responsiveness to light, so that odors from darker places may attract it more readily. On the other hand L. caesar is very strongly phototactic and consequently would seek the open, and if by chance it should find its way into darker places its responsiveness to large luminous areas would soon lead it to escape.   It may be assumed that the two species are equally chemotactic, which assumption is justified at least by observation.   Since C. vomitoria is less strongly phototactic, individuals least so might easily be attracted into fairly dark places, and would not soon be compelled to leave because of their phototropism.

Hence it seems reasonable on the evidence at hand to conclude that because of their different degrees of phototropism C. vomitoria is of more importance as a *household* scavenger (or pest, as the case may be), while L. caesar is distinctly a scavenger of the open fields, lake beaches and the like.

A further inspection of table 7 leads one to conclude, that both species are about equally positive to the larger luminous area, which indicates (if we accept the test as conclusive) that the image forming power of the two species is about equal, with possibly a a slight advantage in favor of L. caesar.

It was shown that the eyeless larvae took a straight path between the two lights without turning, while the adults, which possess compound eyes, turned toward the larger luminous field more frequently by 26 to 41 percent (excess over smaller), which indicates discrimination. Since the intensity of the two lights was equal the discrimination must be due to the size of the luminous field and the consequent image formed in the eye. Therefore, these experiments afford a test of image forming powers.

How do the results from the sarcophagid flies agree with those from Vanessa antiope? Cole ('07, pp. 380–382) found 87.2 per cent of the responses of Vanessa were toward the larger light, an excess of 75 per cent of the whole. Though the ratios of the two lights in Cole's experiments (1:10000) were not the same as those in mine (1:7500), it seems quite likely that the eyes of the mourning cloak butterfly are better adapted to the formation of images than the eyes of the sarcophagids. The flesh-flies from the nature of their habits are probably more dependent on odors and would necessarily not need image forming eyes of a very perfect character. Observations made by other investigators further justify the findings of Cole; *e.g.* Parker ('03, p. 461) calls attention to the manner in which Vanessa alights with spread wings and is thus found by other individuals of the same species, involving a well developed image-forming power. Also Latter ('04, p. 88) "once observed a Brimstone butterfly visiting flowers of the Dog violet scattered along a bank, and picking out these flowers to the exclusion of all others with great precision, not even approaching other blue flowers that were present."

The fact that Vanessa antiope alights in sunny spots, as shown by Parker ('03, p. 461), is correlated with its reaction to luminous areas, and similar observations made on Lucilia caesar by the writer lead to a similar conclusion. It was frequently noted that when a dead fish was placed under a leafy tree so that a patch of sunlight fell upon it, flies would soon be hovering about the carcass. As the patch of sunlight left the fish, the flies also disappeared. This behavior was noted, and afterwards the fish was always placed in a patch of sunlight or in the open, and not in a position where shade and patches of bright light intermingled. This condition does not hold true in large shady areas produced, for example, by a building or other large object where there is an absence of sunlit patches.

The experiments with reference to image formation in the sarcophagid flies are of further interest also because these observations place at least this family of Diptera in line with other groups worked on by Cole ('07), who was, however, unsuccessful with his experiments on the fruit fly, Drosophila ampelophila.

IV. APPLICATION TO GENERAL THEORY OF ANIMAL BEHAVIOR

During the progress of the experiments described and discussed in the preceding pages, it was a matter of concern to analyze the movements of the flies in order to ascertain their method of orientation and the factors involved. The matter for consideration in the following pages may be briefly stated in the form of three questions, viz., (A) How is the animal *oriented by light*? (B) How does the animal *orient to light*? (C) Why is there this behavior toward light? The first question is concerned with external, the second and third with internal factors.

A. How is the animal oriented by light? Sarcophagid fly larvae are stimulated to motion by light and that motion is away from the source of light.

As early as 1853 the relative importance of intensity and direction of light were made the object of some observation, at which time Cohn ('53) pointed out that Stephanosphaera collected in relatively darker situations and avoided bright light. Later this

author (Cohn, '66, p. 164) advocated the view that the direction of the rays rather than intensity was the more important factor. "Weitere Versuche haben jedoch erwiesen, das nicht die Intensität sondern die Richtung der Lichtstrahlen es ist, welche die Bewegungen der mikroskopischen Organismen beherrscht."

From that time on investigators have favored one or the other of the two views. Davenport ('97, p. 211) designates the effects produced by the direction of the ray as phototactic and that produced by the "difference in illumination of parts of the organism" as photopathic.

It is quite evident that both phototaxis and photopathy play an important rôle in the movements of the flesh-fly larvae. The path of these individuals is largely pre-determined by the direction of the rays, its response being phototactic in that respect, while, on the other hand, the creeping animal keeps its head as far as possible in the shadow of its own body,—a photophathic response. This statement, based on the evidence already discussed, is in direct opposition to the conclusions of Holt and Lee ('01, p. 462) who say: "Experimental study and a review of the literature on the subject have convinced us that the phenomena thus far reported do not demonstrate either that direction of ray and intensity of light operates separately, or that any distinction should be made between phototaxis and photopathy as independent forms of irritability," and further (p. 479) "The direction of the rays has, in itself, no effect whatsoever, on the movements of the organism." The conclusions reached by Walter ('07) on planarians is more or less in agreement with the conclusions of these authors. Walter states that, in general, intensity rather than direction is "the operative factor in light reactions," but he modifies this by the further statement that "At the same time there is much evidence that the intensity utilized by the organisms, is intimately associated with, and powerfully modified by the direction of the light." These conclusions of Walter are also in accord with those of Loeb ('93, p. 101) on another species of planarian (Planaria torva), which is not phototactic, but reacts in a very striking manner to changes in light intensity ("unterschiedsempfindlich"). On the larvae of Calliphora vomitoria, however,

Loeb's ('90, pp. 70.71) conclusions are different, as the following quotation shows: "so konnte ich auch für die Muscidenlarven nachweisen, dass sie unter dem Einflusse der Richtung der Strahlen gezwungen waren, auch von Stellen geringer Lichtintensität in solche von höher Lichtintensität zu gehen."

It is perfectly clear that sweeping statements with regard to the action of intensity or direction of the rays cannot be made. Whereas one species of organism is almost exclusively influenced by the intensity, as in planarians, a second group of organisms is chiefly influenced by the direction, as in the sarcophagid fly-larvae, and in both cases the two factors are more or less involved in each other. Furthermore, as has been pointed out by other workers (*e.g.* Walter, '07, pp. 144–145), factors of a more or less disturbing or obscuring nature enter into behavior in general. Thus, there is an element entering into the behavior of the flies under consideration which has received little or no attention by former investigators either in fly-larvae, or in other lower organisms, namely the *after-effects* of light stimulation. For at least 15 to 20 seconds after the light has been turned off, the larva of a flesh-fly may continue creeping in a straight line without an increase in the number of random movements. This phenomenon is well illustrated by the following series of figures (figs. 16–23 inclusive). In all cases the path of the larva was in total darkness for a period of from 15 to 20 seconds preceding the change in direction due to the sudden illumination from in front. The after-effects are still more clearly demonstrated by figures 6–10 inclusive. The trails of the larvae in figs. 6 and 7 are sharply deflected and the succeeding paths are in a new direction and as straight as though the larvae were perfectly oriented to a continuous bilateral stimulus. In figs. 8–10 the movements of the animals are again influenced by the after-effects. One would expect the path of the individual to make a sharp angle at the edge of the more intense field and to take up at once a course parallel to the rays of the directive light. Instead, the path is in its beginning diagonal, plainly influenced by the after-effects of the more intense light.

Clearly, this element must have a profound influence on the behavior of the individuals. Certainly, the seeming indifference to light on the part of the individual whose trail is reproduced in fig. 15, is nothing more than the result of the after-effects of previous stimultion, since the trail soon conforms to the new direction of the light rays. Using this individual as an example, it is to be noted that the larva was perfectly negatively phototactic, moving away from the light in the direction of the arrow. The directive light was turned off when the larva was at the point X, and thence to the point Y the larva continued on its way in total darkness undirected save for the after-effects; at Y the larva was suddenly illuminated by directive light from in front. Evidently regardless of the new stimulation it continued on its way (except for an increase of random movements) directly toward the light. At this juncture there is an apparent response, which, unless the history of the case were known, might be interpreted as positive phototaxis. The real conditions are obscured by the after-effects.

Though the experimental evidence is not sufficiently complete to warrant a general statement, it appears from the observations made, that the after-effects are (within certain bounds) proportional to the intensity which produced them.

This phenomenon is accordingly one which has an obscuring effect, comparable to that of mechanical stimulation referred to on p. 191, and noted in other organisms by several investigators, among them Towle ('00) for Cypridopsis, Holmes ('05b, p. 319) for Ranatra, and Walter ('07, p. 130) for planarians.

B. How does the animal orient to light? The sarcophagid flylarvae orient negatively to light and move away from the source, following very precisely the path of the rays. This behavior is also adhered to even when the course lies in a field of light increasing in intensity in the direction taken by the creeping larva, resulting, however, in uncertainty of orientation and a consequent irregular path.

Two distinct stages may be recognized in the process of phototaxis after *stimulation*; first, *orientation*, which may be direct or

indirect; and, secondly, *locomotion*, a movement toward or away from the source of light depending on the existing relation between the organism and the stimulus.

Many organisms are subject to stimulation on being illuminated, but fewer respond by orientation to the light. Of these (disregarding reactions to bright patches of light and intensity alone) the larger number by far move either toward or away from the source.

The principal concern relates to the two stages in the phototactic process after stimulation and may be expressed by the question: How does the animal orient, and after it is oriented how does it continue on its relatively straight path toward or away from the light?

Three theories based on reflex responses of the organism have been advanced to explain the method of orientation. The oldest of these theories is the *tropism theory* of Verworn ('95, pp. 419–446) and Loeb ('97, pp. 439–441), advanced by the latter as an application of Faraday's conception of lines of force. It is defined by Loeb ('06, p. 140) as follows, "the animal is turned automatically until symmetrical points of its surface are struck equally by the lines of force. As soon as this occurs the animals must keep this orientation, and therefore have no further choice in the direction of their motions."

The second theory is that of Jennings (the *method by trial error*) which is not entirely new, but in its present application is distinctly in advance of previous views. The following extracts from the writings of Jennings ('04, p. 237) will serve to define the theory. "On receiving a stimulus that induces a motor reaction, they try going ahead in various directions. When the direction followed leads to a new stimulus, they try another, till one is found which does not lead to effective stimulation" (p. 252). "This method involves many of the fundamental qualities which we find in the behavior of higher animals, yet with the simplest possible basis in ways of action: a great portion of the behavior consisting often of but one or two definite movements, movements that are stereotyped when considered by themselves, but not stereotyped in their relation to the environment. This method leads upward, offering

at every point opportunity for development, and shows even in the unicellular organisms what must be considered the beginnings of intelligence and of many other qualities in higher animals."

The third theory (the *method by random movements*)is that of Holmes and is, as its author suggests (Holmes, '05a, p. 106), "a form of the trial-and-error method minus the element of learning by experience." it is also regarded as "more indirect." Its definition (p. 102) is briefly as follows. "Of a number of random movements in all directions only those are followed up which bring the animal out of the undesirable situation."

How well this latter method of orientation fits the case may be seen by an examination of figs. 16 to 25. Not less may be said of the second method, indeed, as has already been maintained by the writer (Herms '07, pp. 80–81), there is so little difference between the two methods in their application to fly-larvae, that they might be regarded as equally applicable were it not for the qualifying statement of Holmes "minus the element of learning by experience." There seems little ground for doubting that either the second or third methods find their application in the behavior of these organisms under the given intensities (figs. 16, 17, 20, 21, 22, and 25); but increasing the intensity lessens very decidedly the number of random movements, as illustrated by figs. 18, 19, and 23. This result is in accordance with the statement of Loeb ('88, p. 3) that the orientation of the animal in the direction of the rays is more precise as the intensity increases. It was also found by Harper ('05, p. 17) in the earthworm (Perichaeta bermudensis) that "random movements are a feature of less strong light, tending to disappear with the increase of intensity, and are replaced by direct orientation in very strong light."

To test the influence of a range of intensities on the production of random movements, the larvae were started in a very low directive intensity (0.5 to 1.0 C.M.), and after the individuals had oriented and were crawling away, the directive light was turned off leaving the larvae to creep in total darkness for 15 to 20 seconds, when a new directive light from in front of the animals was suddenly turned on. It may be seen by the courses illustrated in figs. 16 to 23, that sudden darkness had no apparent effect on the
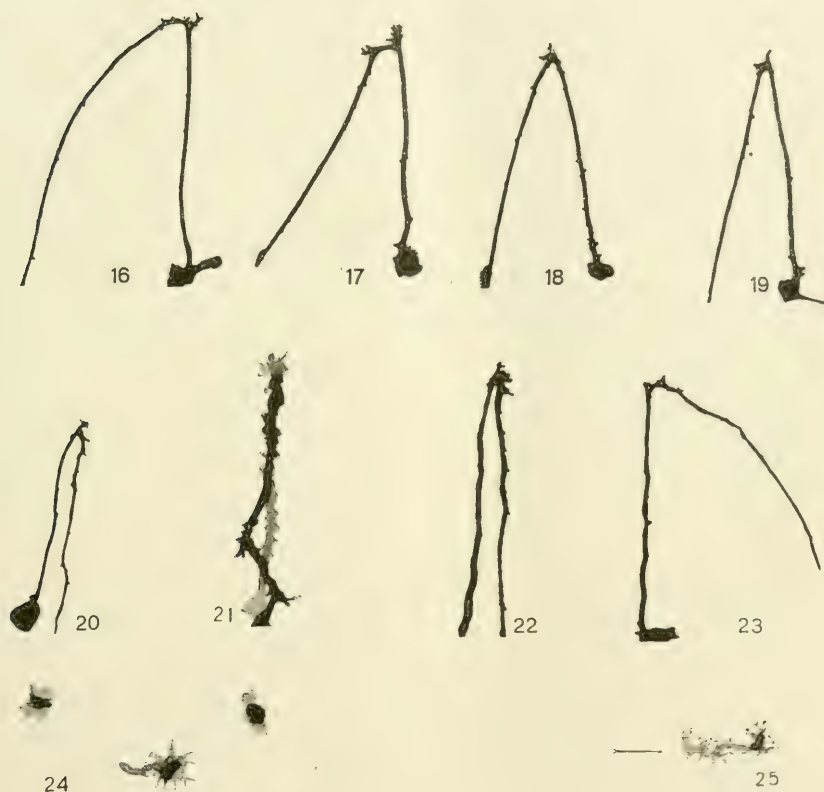
Fig. 16 L. caesar. Random movements under an intensity of 0.007 C. M.
Fig. 17 L. caesar. Random movements under 0.56 C. M.
Fig. 18 L. caesar. Random movements under 325 C. M.
Fig. 19 L. caesar. Random movements under 960 C. M.
Fig. 20 C. vomitoria. Random movements under 0.56 C. M.
Fig. 21 C. vomitoria. Random movements under 64 C. M.
Fig. 22 C. vomitoria. Random movements under 325 C. M.
Fig. 23 C. vomitoria. Random movements under 960 C. M.
Fig. 24 Ramdom movements of C. vomitoria in total darkness for one minute. The larva is undirected. Rest is the usual state under such conditions, but mechanical stimulaton due to handling caused the larvae to be restless and to produce random movements.
Fig. 25 C. vomitoria. Randon movements under 0.14 C. M.

larvae, quite the reverse from a sudden increase of illumination. For C. vomitoria, where the after-effects were so pronounced in experimenting with low intensities, no results were obtained to illustrate this principle in the same manner, but fig. 25 is substituted and represents the matter quite as clearly. The courses seen in this series of figures (fig. 16 to 25) show without further explanation that the random or trial movements are characteristic for low intensities, and become fewer with the increase in intensity until finally the orientation is to all intents and purposes direct, *i.e.*, the animal turns directly away from light of high intensity. Thus there is almost a perfect gradation between the indirect method of random movements and the direct tropism scheme of behavior with regard to orientation. Therefore it seems preferable in reference to the organisms in question to designate this as a *combination* method of orientation, uniting the two general schemes in one. The larger number of random movements produced by C. vomitoria is again indicative of its lower degree of sensitiveness as compared with L. caesar.

With the orientation of the animal to the source of light there is still to be considered the second step in the process of phototaxis, *i.e.*, the movement away from the stimulus. This step is accomplished, as is evident from an examination of any of the figures, by the action of the light rays operating bilaterally on the organism, it *must* keep its general direction. Any deviation from this predetermined path is, however, corrected by trial movements in a more restricted sense. The circus movements produced by larvae when one side of the head is pigmented is further evidence that these organisms when once oriented follow the direct or tropism scheme of behavior.

C. Why is there this behavior to light? It has been shown that the sarcophagid fly-larvae respond to light, orient negatively and creep away from the source of light, in short are negatively phototactic. What is the relation between this behavior of the larvae and the conditions under which they exist normally?

It will be recalled that the eggs of the adult females are deposited on dead animals found in light situations. The larvae while feeding are largely protected from light and birds by the carcass,

but as the moisture is eliminated and the larvae become full grown they are left in a rather precarious position. The remnants of the fish or other dead body can then be easily dislodged by the winds or other mechanical means, and the larvae are then exposed to the ravages of birds, as has already been stated. The heat of the sun also would then result disastrously to them. The importance of moisture in the economy of the flesh-flies has been pointed out on several occasions (Herms, '07, pp. 49, 67, and 75).

It must be evident that the phototactic response of these larvae is highly adaptive to the situation. By this means the larvae find places of shelter from light, away from the desiccating influence of the sun, removed from preying birds and other disturbing elements. This response, coupled with their strong thigmotactic reaction (Herms, '07, p. 82) as seen in burrowing, affords a natural and almost complete protection for the individual and consequently for the species.

V. GENERAL SUMMARY

1. The egg-stage of Lucilia caesar and Calliphora vomitoria covers a period of from 8 to 48 hours; the feeding period from 2 to 7 days for the former species, and from 3 to 9 days for the latter; the prepupal period usually from 2 to 7 days (extreme 59 days); the pupal period usually from 8 to 17 days (extreme 34 days). The most variable in length is the prepupal period. Constancy in environmental conditions results in uniformity. The shortest time observed between emergence from the pupa and egg deposition was 9 days for Lucilia caesar and 12 days for Calliphora vomitoria.

2. The usual length of life of an imago flesh-fly is about 30 days, regardless of sex. The longest recorded life of a single adult individual was 63 days.

3. A positive reaction to kerosene lamplight, acetylene gaslight, monochromatic light, and solar spectrum was demonstrated on the part of the aggregated feeding larvae of Lucilia caesar during a limited part of this period, causing the larvae to leave their food (as far as 16 cm.). Under the solar spectrum the feeding larvae are positive to the red end, collecting especially in the yellow-

orange band when given the entire range of the spectrum. Sudden increase or decrease in intensity, jarring, or changing lights suddenly, caused the larvae to abandon temporarily the positive relation to light.

4. The migrated larvae of Lucilia caesar are negative to any color of the spectrum when the color is used singly, but they will collect on the side away from the source of the light in the yellow-orange band when the entire range of the spectrum is available. The blue end of the spectrum is strongly avoided.

5. The larvae of both species as individuals react negatively to directive light throughout this stage, but are not strongly responsive on hatching, becoming more and more markedly so as they grow older. They are also strongly reactive to shadows, *i.e.*, are photopathic.

6. The imagoes emerge from the pupal stage as organisms positive to light.

7. The lowest directive intensity to which the larvae of Lucilia caesar respond is 0.00076 C.M., while for Calliphora vomitoria it is 0.007 C.M.

8. Below the respective minimum directive intensity, light still has a dynamic effect until an intensity of 0.00007 C.M. is reached, when the conditions are equal to darkness, *i.e.*, the larvae remain at rest.

9. The courses of the larvae under stimulation from two sides always lay more or less transverse to the light rays, the greater deflection being toward the light of lower intensity regardless of the size of the luminous field.

10. When placed midway between two balanced lights of like luminous areas (or unlike areas, but like intensity) their course lay midway between the two lights, transverse to the direction of the rays.

11. There is no constant relation between the movements of the larvae and a graded field of non-directive light.

12. With a proportionately high directive intensity, the larvae passed directly into and through a field of higher intensity (non-directive), but when a proportionately low intensity was used the

larvae could seldom be induced to enter a field of high intensity (non-directive).

13. By means of a low intensity of directive light (0.5 C.M.) the larvae could be forced farther into the darker end of a field grading at the rate of 7 C.M. per cm. than into one grading at the rate of 35 C.M. per cm. Though the relation was not constant, the usual distance was about five times as far in the former field.

14. In a field of light which is at the same time directive and graded to higher intensities in the direction of the rays, the larvae creep with the rays, thus passing into more and more intense regions, not, however, without a disturbance in orientation, indicated by many random movements and a strong tendency to take a diagonal course with relation to the rays.

15. In comparison with the records of other investigators, it appears that more perfect orientation is possible on the part of the fly-larvae because of the localizating of the photoreceptive organs.

16. The rate of locomotion increases rather uniformly with the intensity of the light, excepting for the lower and higher extremes.

17. The increase in rate in higher intensities is not alone due to precise orientation, but also to photodynamic effects.

18. By sudden increase in illumination, whether the individuals were previously creeping in the dark or in 0.5 C.M. or in 50 C.M., the larvae invariably produced random movements at the moment and were more or less thrown out of orientation. The reverse condition (sudden decrease of illumination) did not result in any preceptible change of state at the moment.

19. The functional photoreceptive organs are highly concentrated and equally distributed bilaterally in the immediate region of the oral pole.

20. The more frequent presence of the adults of C. vomitoria in houses and like situations may be accounted for by the fact that this species is less responsive to light and also less strongly phototactic than is L. caesar. Therefore the former is of more importance as a household scavenger or pest, as the case may be, while

the latter is distinctly a scavenger of the open fields, lake beaches and like situations.

21.   The adults when tested for opposing fields of different areas but with like intensity react positively to the larger area of light.

22.   The image forming powers of the eyes of the adults are about equal for the two species, and relatively well developed.

23.   The path of the individual larvae is largely predetermined by the direction of the rays, but is greatly influenced by intensity.

24.   The *after-effects* of light stimulation are an important modifying or obscuring factor in behavior, as is also mechanical stimulation.

25.   In the process of phototaxis after *stimulation*, at least two distinct steps should be recognized, first, *orientation*, leading to, second, *locomotion*.

26.   In low light intensities these larvae orient by an *indirect* method, which may be termed "trial and error" or "random movements;" in higher intensities the larvae orient by a *direct* method.

27.   There is a gradual transition from one method to the other; therefore it seems preferable in reference to the organisms in question to designate this as a *combination method of orientation*, uniting the two general schemes in one.

28.   The locomotion of the organisms away from the source of light is governed almost exclusively by the influence of symmetrical stimulation on a bilateral animal, and consequently falls under the definition of a tropism.

29.   The negatively phototactic reaction of sarcophagid fly-larvae is highly adaptive.

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REACTIONS OF BRANCHIPUS SERRATUS TO LIGHT, HEAT AND GRAVITY

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In the composite of activities which we call the habits of Branchipus serratus, various reactions to external stimuli, such as light, heat and gravity may be assumed to occur. The present paper records a number of laboratory experiments designed to test under controlled conditions certain of these reactions, and thus obtain more information as to the rôles played by the three environmental factors in the daily life of the crustaceans.

BEHAVIOR WITH RESPECT TO LIGHT

Phototropisms with light of different intensities

Several years ago while engaged in a laboratory study of this species, the writer's attention was called to the great readiness with which Branchipus reacted positively to light areas of considerable difference in intensity. When kept in a glass aquarium in diffuse light there was only a slight tendency on the part of the animals to collect on the side next the source of light; but when a screen was erected between one-half of the aquarium and the light, in such a way as to make a light portion and a shaded portion, the animals swarmed out from the shaded area into the light one. If two screens were set up, making two shaded portions with a light streak between them, the animals collected in the light streak.

A series of experiments was carried on in the spring of 1910 to test this positive reaction under more carefully controlled condi-

tions. It was desired (1) to learn whether, under the influence of lights of different intensities, the response remained positive, and (2) to find whether exposure to darkness had any effect on the character of the response.

For the first experiment, a dark box, 4 feet wide, 3 feet high, and 2 feet deep was constructed, and a 12 c.p.[2] incandescent lamp was suspended from the top of the box. A rectangular black pan was placed on the floor of the box, about 2 feet from the lamp. This pan was provided with a black cardboard cover having a central opening, 2 inches square, directly under the light. The pan was filled with water to a depth of about 2 inches, and eight specimens of Branchipus were dropped in at one corner. When the cover was put on the pan, all the animals came very quickly into the light space.

When once trapped here, they swam about at random until they came in contact with the shadow, to which they immediately responded negatively, and so remained in the lighted area. The shadow was as effective a barrier as a stone wall would have been. The experiment was repeated many times, always with the same result. When the opening in the center was closed, and an opening made in the corner of the cover, the light remaining above the center, the animals went to the lighted corner.

The specimens were next placed in a rectangular glass aquarium in the dark box. A 12 c.p. incandescent lamp, which was movable, was placed at the end of the aquarium. The animals immediately swarmed into the end next the light. When the light was moved to the opposite end they turned and again swam toward it. As often as the light was changed, the animals changed their course accordingly. With the specimens in a tall glass cylinder, and the light above or below, the same positive reactions were obtained.

These preliminary experiments show that Branchipus is positively phototropic to a 12 c.p. light.

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[2] The candle powers given in this paper are from actual photometric measurements of the electric lights used; but since the strength of the current varied considerably from time to time the figures are at least only approximate. Generally speaking c.p. as used in the text corresponds to *candle meters* but the conditions of the experiment do not permit an exact statement in regard to light values.

Since certain investigators (Parker, '02; Juday, '04; and others) have shown that some crustaceans are positive to weak light while to light of higher intensities they are negative, it seemed that the same might be true of Branchipus. Accordingly they were tested with all the different intensities available, 12, 25, and 280 candle powers.

The 25 c.p. light was used in the same way as the 12 c.p. light employed in the previous experiment. The same rectangular glass aquarium was placed in the dark box, and the light changed from end to end as before. The specimens responded positively as with the weaker light.

The 280 c.p. light was an arc lamp suspended in a dark room. The aquarium was raised until it was on a level with the light as in the previous experiments. Since the light could not be changed from one side of the aquarium to the other, the latter was turned around when the animals collected in the illuminated end. To this arc light the animals were uniformly positive both in the aquarium and in the pan with the cardboard cover containing the square opening.

From the results of these experiments, Branchipus may be considered positively phototropic to lights varying in intensity from 12 to 280 c.p. These results agree with those of Yerkes ('01) for Daphnia, and of Carpenter ('05) for Drosophila.

An interesting observation in connection with the above experiments was made on the body orientation. Ordinarily, in an aquarium or in ponds, Branchipus is seen swimming with its ventral side up; but with the aquarium in the dark box, where we can control the source and direction of the light, a different position of the body may usually be observed. When, with the light at one end of the aquarium, an animal is dropped in at the darker end, it immediately starts toward the light, with the ventral side directed toward it, and the long axis of the body at right angles to the light rays. If the light is changed to the opposite side, the animal turns and assumes the same orientation. If the light is placed above or below a tall glass cylinder used as an aquarium, Branchipus swims upward or downward, with the ventral side always directed toward the light. It is only occasionally that an

animal, swimming rapidly toward the light, directs the head forward, and places the body parallel with the light rays.

A superficial examination of the eyes with a dissecting microscope does not reveal any unequal distribution of the pigment to account for this peculiar orientation of the body with respect to the source of light.

Effect of previous exposure to darkness

Two sets of experiments were undertaken to determine the effect of more or less prolonged exposure to darkness on the character of the phototropic response. One series was intended to test animals that had been subjected to short exposures of from twelve to fifty-six hours. The other series was concerned with animals reared in the dark from early embryonic stages.

After 12 hours in the dark the two specimens experimented with reacted to the light in a normal way. After an exposure of twenty-eight hours, four other specimens responded in the usual manner. Of four males exposed to darkness for 56 hours, three were positive to the light, while one appeared negative.

In the case of four specimens reared in the dark, it was found that they responded positively to light just as do those reared under ordinary conditions of illumination.

It seems clear from these observations that previous exposure to darkness does not change the positive phototropism. The single exception mentioned above is probably referable to another cause. When the animals were moulting they were very inactive, responding very slowly, not at all, or negatively.

Kinetic effect of light

Branchipus seemed to move faster in bright than in less intense light, and experiments were made to test more carefully this apparent photokinetic response.

One of the most noticeable of the movements of Branchipus is the constant vibratory motion of the swimming feet. Whether the animal is swimming or lying quiet, its feet are continuously engaged in this motion, which is more rapid when the animal is

moving about than when at rest. The movement is wave-like, progressing from the posterior feet to the anterior. The swimming seems to be accomplished by this motion and the bending of the abdomen, the abdomen and telson together acting as a rudder. The fact that the beating to and fro of the swimming feet has a respiratory as well as a locomotor function was brought out in a series of experiments performed in this laboratory, in which the rapidity of the beats was shown to increase with the addition of CO_2 to the water until a certain limit was reached, when the further addition of CO_2 caused the oscillations to cease entirely.

It was thought that by counting these vibrations in different light intensities some quantitative data might be obtained, which would aid in answering the question as to the kinetic effect of light on the swimming appendages. Although the data obtained were insufficient in quantity to be conclusive, nevertheless they seem to indicate that the activity of Branchipus increases with an increase in the intensity of the light.

For the purpose of counting the vibrations of the swimming feet the glass aquarium was placed in the dark box directly under the 12 c.p. light, and a heat screen was interposed between the light and the aquarium. The heat screen consisted of a circular glass dish filled to the depth of one inch with water, which was frequently changed. Since the light was practically non-directive under these conditions, the animals swam about at random, or lay quietly on the bottom of the aquarium. The vibrations of the swimming feet in a given time could be easily counted. A 25 c.p. light was used next under similar conditions, and counts were made as before. The next day the experiment was repeated in the reverse order, the 25 c.p. light being used first. Since males were found to react to the light somewhat more quickly than females, and since individuals in the process of moulting seemed to be less responsive, the same individual was used during an experiment with the two lights. The results are given in tables 1 and 2.

Further trials were made with three individuals, using the 280 c.p. arc light. These did not show any increase in the number of vibrations per second over the number recorded for the 25 c.p. light. However, these individuals died the day after the

TABLE 1

12 c.p. light

MALE NO. 1	TIME IN SECONDS	NO. OF VIBRATIONS	NO. OF VIBRATIONS PER SECOND
Trial No.			
1	69	185	2.9
2	63	154	2.4
3	60	158	2.6
4	81	200	2.6
5	60	156	2.6
6	60	165	2.68
7	61	148	2.4
8	60	155	2.58
9	60	150	2.5
10	60	156	2.6
Average			2.58

25 c.p. light

MALE NO. 1	TIME IN SECONDS	NO. OF VIBRATIONS	NO. OF VIBRATIONS PER SECOND
Trial No.			
1	60	196	3.2
2	60	167	2.8
3	63	193	3.
4	63	187	3.
5	60	173	2.88
6	65	193	3.
7	63.6	181	2.9
8	74	209	2.8
9	68	190	2.8
10	62.8	172	2.8
Average			2.91

experiment, and an examination with the microscope showed them to be infected with a parasite. Their behavior, therefore, could not be accepted as normal. The Branchipus season being shorter than usual, no more material could be procured, and consequently the experiment could not be repeated.

TABLE 2

25 c.p. light

MALE NO. 2	TIME IN SECONDS	NO. OF VIBRATIONS	NO. OF VIBRATIONS PER SECOND
Trial No.			
1	60	175	2.9
2	60	190	3.1
3	60	178	2.96
4	60	188	3.1
5	60	184	3.
6	60	173	2.9
7	60	189	3.1
8	60	183	3.
9	61	182	3.
10	61	177	2.9
Average...			2.99

12 c.p. light

MALE NO. 2	TIME IN SECONDS	NO. OF VIBRATIONS	NO. OF VIBRATIONS PER SECOND
Trial No.			
1	61	157	2.5
2	61	159	2.6
3	60	166	2.76
4	60	151	2.51
5	61.8	163	2.63
6	60	165	2.75
7	60	162	2.7
8	64.4	181	2.81
9	60.4	174	2.88
10	72.2	190	2.63
Average...			2.67

Photoreceptors

An effort was made to remove the eyes, in order to determine whether any other part of the body is sensitive to light. The operation however, though very carefully performed, proved fatal to the animals. Recourse was then made to covering the eyes with an opaque cap of Canada balsam and lampblack. The specimens were laid on a moist filter paper under a dissecting lens and after the moisture had been removed as completely as possible from the eyes, the balsam and lampblack were applied. The operation was entirely successful with only two individuals. These were returned to the water and allowed to recover. It was found that they no longer responded to the light, but rose to the surface and stayed there. The upward movement was not due to the low specific gravity of the balsam, for animals which died with the balsam on the eyes sank to the bottom. This reaction will be discussed further under the account of the influence of gravity.

We may conclude from these results that the eyes are the only sense organs for the perception of light.

BEHAVIOR WITH RESPECT TO HEAT

Reactions to increased and decreased temperatures

In order to study the temperature reactions of Branchipus, the large pan with black bottom and sides was placed under a 12 c.p. lamp, suspended from the ceiling of the dark room at such a distance that the light was practically uniform in its distribution, and hence had no directive effect upon the horizontal movements of the animals.

Ice was placed in one corner of the pan, and the temperature reduced immediately under the ice to 8° C. The corner diagonally across from this was heated to 30°. Seventeen animals were put in at the warm corner, and in a short time they were distributed as follows: seven in a region where the temperature was 16.5°, five where the temperature was 17°, three where the temperature was 12°, and two under the ice in the region of 8°. There were none in any region warmer than 17°. The animals did not remain distributed in this way, but the greatest number moved about

between the temperatures of 14° and 17°. They were kept in the pan thirty minutes, and during that time they stayed out of regions where the temperature was higher than 17° C.

Various other tests of the behavior of Branchipus to different temperatures were made with results confirming those just given. From 14° to 17° C. may, therefore, be considered the optimum temperature for the majority of the animals tested, that is, the temperature chosen by them when free to move into either warmer or cooler water. When confined in water as warm as 28°C. or above Branchipus soon perishes.

BEHAVIOR WITH RESPECT TO GRAVITY

Reaction to gravity in light

When exposed to diffuse light these crustaceans are usually to be seen lying at rest on the bottom of the aquarium, or quietly swimming near the bottom. When the animals were put into distilled water in tall glass cylinders for a more critical test of what seemed to be their positive geotropism, they went down at once, and stayed near the bottom as long as the light was diffuse enough to be non-directive.

An experiment was devised to test the behavior with respect to gravity in directive light. A glass cylinder, twelve inches high and six inches in diameter, was used. It was filled with distilled water thoroughly aërated, and was then placed vertically in the dark box between two 12 c.p. incandescent lamps, one above and one below, each being about 6 inches from the cylinder. When the light above was turned on, the animals were attracted upward toward it, and a record was kept of the time required to make the trip. When the light was turned on below the animals traveled downward, and a record was kept as before. Records were also taken of trips made under similar conditions with the cylinder lying horizontally on the floor of the box, and the lights 6 inches from the ends. Numerous tests were made with different individuals on different days, the results in all cases being similar. Two typical laboratory records of this kind are given below, one for the cylinder in a vertical position, and one for the cylinder in a horizontal position.

APRIL 20, 1910. MALE		APRIL 20, 1910. MALE	
1st trial................	Up, 180 sec. down, 10 sec.	1st trial..........	To left, 30 sec. to right, 40 sec.
2nd trial................	Up, 90 sec. down 8 sec.	2nd trial.........	To left, 20 sec. to right, 20 sec.
3rd trial................	Up, 85 sec. down, 22 sec.	3rd trial.........	To left, 30 sec. to right, 20 sec.
4th trial................	Up, 80 sec. down, 15 sec.	4th trial.........	To left, 20 sec. to right, 20 sec.
5th trial................	Up, 65 sec. down, 25 sec.	5th trial..........	To left, 13 sec. to right, 12 sec.
6th trial................	Up, 15 sec. down, 12 sec.	6th trial..........	To left, 30 sec. to right, 20 sec.
Average...............	Up, 85.8 sec. down, 15.3 sec.	Average........	To left, 23.8 sec. to right, 22 sec.

An examination of these records reveals that Branchipus travels downward in response to light more rapidly than upward, while its rate of movement horizontally is about the same toward the left as toward the right. The influence of gravity on the rapidity of its locomotion in vertical planes is evident.

It is to be noted that distilled water was used in all the experiments concerned with gravity. This eliminated the possibility of chemotropic reactions to foreign materials at the bottom of the containers. Such reactions may be suspected to occur in an ordinary aquarium in which deposits of débris are present.

While the observations recorded above seem to warrant us in holding that Branchipus is positively geotropic when exposed to light, the methods used in obtaining the reactions do not enable us to decide whether we are dealing with a definite orientation with respect to the center of the earth, or with the purely mechanical effect of gravity. It may be that the readiness with which the animals seek the bottom is to be explained by their tendency to sink in water.

Reaction to gravity in darkness

During the progress of the foregoing experiments it was noticed that when specimens were left in the dark box with the curtain

down, and the lights turned off, they were always found at the surface of the water. When the curtain was raised, and non-directive light admitted, they immediately went to the bottom. Accordingly more critical tests were made of this apparent reversal of response to gravity.

The glass cylinder was graduated into six 2.5 cm. divisions, and filled with distilled water to the top of the first division. A cardboard box of suitable size blackened on the inside, was used as a cover for the cylinder.

When the animals were placed in the cylinder in diffuse light, they remained at or near the bottom, but when covered they rose to the surface, in nearly every case. In a few instances, they stayed at the bottom, once when the specimen was observed to be moulting, and at other times when the animals were inactive for causes not clear to me. They did not always rise the whole distance to the surface, but as may be seen by a perusal of the record given below, they did so in the majority of cases. The animals did not often seem to swim down, when the cover was removed, but in most instances they apparently sank passively to the bottom. The positions of the specimens, together with the time of covering and uncovering, are shown in the following record:

April 15, 1910.   One male

8:20. At bottom—covered
8:25. Uncovered—at surface—dropped down immediately
8:30. At bottom—covered
8:35. Uncovered—in division 3; went to bottom immediately
8:37. At bottom—covered
8:42. Uncovered—at bottom—moulting

April 18, 1910.   Three males

8:25. All at bottom—covered
8:35. Uncovered—two at surface, one in division 1—went down at once
8:40. All at bottom—covered
8:45. Uncovered—all at surface—went down at once
8:48. All at bottom—covered
8:53. Uncovered—all at surface—went down at once
8:55. All at bottom—covered
8:58. Two at surface—one in division 2—went down immediately
9:00. All at bottom—covered
9:05. Uncovered—all at surface—went down at once

By covering the cylinder with a black cloth through which some light penetrated, a dim illumination was produced. Under these conditions the animals remained at the bottom.

The above record indicates that in darkness Branchipus shows marked negative geotropism. We may conclude that Branchipus, like Cyclops (Esterly '07), is positively geotropic in the light, while in darkness this response is changed to a negative one.

Parker ('02) and Juday ('04), in their investigations concerning daily depth migrations with animals which are negative to ordinary daylight but positive to dim light, account for their rise to the surface at night, by the combined effect of negative geotropism and positive phototropism to weak light. The descent in the daytime, they attribute to negative phototropism in strong light. This response to strong light overcomes the negative response to gravity.

It is probable from the present experiments with Branchipus, and those of Esterly with Cyclops, that gravity, as well as light, does have an important influence upon the depth migrations of these crustaceans; but, at least as far as Branchipus is concerned, not in the manner suggested by Parker and Juday. It seems more probable, in the case of Branchipus, which is always positively phototropic, that light has an effect in addition to its directive and kinetic effects, so that through some internal change, the animal is made positively geotropic, and hence goes down in the daytime. Or it may be that darkness furnishes a stimulus which renders the animal negatively geotropic, and so causes it to rise to the surface in the absence of light. It appears therefore that light (or darkness) brings about a reversal of response to gravity, just as mechanical influence brings about a reversal of response to light, as shown by Towle ('00) and as Loeb ('04) has shown chemical influence to cause a reversal of response to light.

When this investigation was undertaken it was intended to check and supplement the results obtained in the laboratory by field observations on the daily life of Branchipus in nature. On account of the unusual drouth early in the spring of 1910 in the vicinity of Urbana the Branchipus season was shortened by about

two weeks, and opportunities for field observations considerably lessened in consequence. The results obtained were so incomplete that it has been thought best to confine this paper to an account of the experimental work carried out in the laboratory.

I am indebted to Dr. Charles F. Hottes, and Dr. Charles Zeleny for many courtesies extended to me during the progress of the work. Especially do I wish to express my gratitude to Dr. F. W. Carpenter, under whose direction the work was carried on, for his careful supervision and kind suggestions.

SUMMARY

1.   Branchipus serratus is positively phototropic under the influence of lights varying in intensity from 12 to 280 candle power.

2.   After exposure to darkness, varying from twleve hours to six weeks, the animals still respond positively to light.

3.   Light apparently has a kinetic effect which increases with the intensity.

4.   When Branchipus reacts to light the ventral side of the body is usually turned toward the source of light, with the long axis of the body lying at right angles to the light rays.

5.   The eyes are the only sense organs capable of receiving the light stimulus.

6.   Branchipus responds positively to temperatures of from 14° to 17° C., and avoids temperatures above or below this. Temperatures as high as 28° C. are fatal.

7.   The animals are positively geotropic in light, and negatively geotropic in darkness.

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STUDIES ON HYBRID DUCKS

H. D. GOODALE

NINE FIGURES

TWO PLATES

As no studies from a Mendelian standpoint seem to have been made on ducks, the following experiments were undertaken. The results thus far obtained are sufficiently interesting and suggestive to make a first report on the work in progress desirable. The record shows that too few individuals in the F_1 generation have been reared to make much theoretical discussion profitable.

My thanks are due Mr. B. B. Horton for permitting me to carry on this work at "Oakwood."

DESCRIPTION OF BREEDS

The two breeds used, which were crossed reciprocally, were the Pekins and Rouens. It is well known that both varieties breed true. Some 200 of the former and 40 of the latter have been reared, and without exception have been true to type.

The Pekins (fig. 1) are white with yellow bills. The shanks and feet are orange. The ducklings are yellow throughout.

The Rouens are practically domesticated Mallards. The male (fig. 2) is brilliantly colored. His head and the upper half of the neck is deep lustrous green, bounded on its lower edge with a narrow white ring, often incomplete dorsally. The ventral side of the lower half of the neck, together with the breast (*i. e.*, anterior to keel) is a deep claret (maroon). The remainder of the ventral surface is iron gray, becoming black posterior to the anus. The entire dorsal surface, posterior to the neck ring, is dark, being dull brownish anteriorly, becoming black in the

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middle of the back, and greenish black on the rump. All the gray feathers, scapulars, and the anterior dorsal feathers are vermiculated. The main tail feathers are dull black. A speculum of iridescent purple is formed by the major part of the exposed surface of the secondaries. The under side of the wing, not including the remiges, is creamy white. The bill is light greenish yellow. The shanks and feet are orange. The female, (fig. 3), by contrast, is rather plain colored. On the whole she may be described as streaked dark brown and buff. The upper surface is considerably darker than the ventral. The throat and sides of the head are buff with two dark stripes passing across the latter (fig. 4A). There is no neck ring. The speculum and under surface of the wing are the same as in the male. The bill is dull brownish black, with greenish yellow blotches. The shanks and feet are orange. The newly hatched Rouen duckling of both sexes is dull black with two dull yellow stripes on each side of the head and with some dull yellow spots on the body.

THE F_1 GENERATION

Owing to the method of making the matings the individual mothers of the F_1 generation are not known. A Rouen male was placed in a pen of Pekin females and *vice versa*. There were 23 young hatched from both matings, 13 were hatched here in 1909; 6 had Rouen mothers, 7 Pekin. The other 10, all Pekin males by Rouen females, were hatched and reared for me by my brother in 1910. Their parents were from my own stock. Of these I personally saw only 5 females as adults. Besides these there were nearly a dozen others from the same matings, but in which the ducklings from the reciprocal crosses were mixed together owing to an oversight at hatching time.

F_1 ducklings. Part of the F_1 ducklings belonged to the Rouen type. The remainder had a new type of down. These were dull yellow, very different from the Pekin color, and with an under color of dull black which usually came to the surface on the wings and tail. This type probably represents an incomplete dominance of the Rouen down over the Pekin. The only exceptions to these

forms were two black ducklings which appeared among the dozen mentioned above.

F_1 *adults.*   From the mating Rouen female and Pekin male, seven females and five males grew to maturity.   From the reciprocal mating there were two females and three males.   These were all pigmented and on the whole closely resembled the Rouens. When, however, the various characters were considered separately it was found that the hybrids differed in several points from the Rouens, as will appear from the following description.   Besides these there were two greenish black females with white throats and breasts (fig. 7).   They appeared among the lot of hybrids in which the reciprocal crosses were mixed.

The males (fig. 5), generally speaking, are all alike, such variation as was noted being one of degree rather than kind.   The head is identical with that of the male Rouen.   The rest of the body is usually somewhat lighter in tone, so that the hybrids appear brighter than the Rouen male.   There is a neck ring, several times as broad as that of the Rouen in all the males. The claret feathers of the breast are partly white, especially in the midline.   On the other hand, the feathers of the dorsal side of the lower neck contain much claret.   The dorsal surface of the wing usually is quite different from the Rouen.   The anterior third is nearly white, due to the presence of a broad white margin to each feather.   The next three rows or thereabouts have less white, but present a broad submarginal band of rufous, which becomes less conspicuous in the next two rows.   The next row, which lies directly over the remiges, is not especially modified. A variable number of the primaries are always white.   The main tail feathers may have white or vermiculated margins.   Vermiculations may also appear in sections where they do not occur in the pure Rouen.   There is one male, however, in which the breast and the dorsal surface of the wing is the same as that of the thorough-bred Rouen.   This may be merely a variation in dominance, or it may have greater significance.

The females (fig. 6), with the two exceptions already noted, are also brighter colored than the female Rouens, but not more so than what I am informed is the fancier's ideal.   The brighter

color is due to the presence on the feathers of more buff and less black than occurs in the Rouens as I have bred them. The most striking point about the females is that in respect to a given character they can be divided into two classes, viz., those with and those without the character. Some characters, *e. g.*, neck ring, are present or absent in the female irrespective of the direction in which the cross was made. But the data at hand are insufficient to make a statement of any value regarding other characters studied. The important point at present is the existance of two classes of females, in respect to certain characters, while the males all fall into one class. Thus, while all the males have broad neck rings, only part of the F_1 females have any ring at all. Two of the females lack the head stripes (fig. 4B). In addition, there are the two black females. There appear

TABLE 1

F_2 pigmented males

	NOT INCLUDING BLACK		INCLUDING BLACK	
	Like Rouen male	Modified	Like Rouen male	Modified
Head..........................	7	0	8	0
Bill...........................	7	0	8	0
Chin spot[8]..................	6[1]	0	7	0
Neck ring....................	7	0	7	1[6]
Breast.......................	3	4[2]	3	4[2] 1[5]
Claret.......................	6	1[6]	6	2[6]
Rump.........................	4	3[2]	5	3[2]
Tail..........................	0	6[2]	1	6[2]
Under tail coverts...........	1	5[2]	2	5[2]
Belly.........................	4	2[2]	4	3[2]
Primaries....................	0	6[2]	1	6[2]
Upper surface of wing........	3	3[2]	3	3[2] 1[7]
Under surface of wing........	4	2[3]	4	3[3]
Speculum.....................	4	2[4]	4	3[4]
Legs and feet................	6	0	6	1[9]

[1] The difference in numbers is due to the lack of records on one male recorded simply as "like Rouen drake."   [2] With much white.   [3] Pigmented.   [4] Obscured.   [5] Pure white.   [6] Absent.   [7] Black.   [8] The chin spot is a small white spot at the base of the lower mandible. Among the pure Rouens I have seen it only in the males.   [9] Black and orange.

TABLE 2

F₂ pigmented females

	NOT INCLUDING BLACK		INCLUDING BLACK	
	Like Rouen female	Modified	Like Rouen female	Modified
Head.........................	9	2^1	9	2^1 2^8
Bill.........................	9	2^2	11	2^2
Chin spot	6	5^3	6	7^3
Neck ring....................	5	6^3	7	6^3
Breast.......................	10	1^4	10	1^4 2^9
Belly........................	7	4^4	7	5^4 1^8
Primaries....................	4	7^4	4	9^4
Upper surface of wing........	8	3^4	8	3^4 2^8
Under surface of wing........	9	2^5	9	4^5
Speculum....................	9	2^6	9	4^6
Legs and feet................	7	4^7	7	4^7 2^{10}

[1] Plain.   [2] Nearly yellow.   [3] Present.   [4] With much white.   [5] Pigmented.   [6] Obscured.   [7] With some black.   [8] Black.   [9] Pure white   [10] Black and orange.

also to be several other characters which are present in part of the females and absent in the remainder, but which are either always present or always absent in the male. I am not prepared at present to speak definitely in regard to these characters mainly because this peculiarity in their inheritance was not noticed until the F₂ generation was studied (tables 1 and 2). On the other hand, there are characters which are in only part of the individuals, of each sex. Thus, white shading on the breast and the white-red dorsal wing surface, as described for all but one of the F₁ males, are present in only part of the females. But I have failed to find any character, which is present in only part of the males, but which is always present or always absent in all the females. The significance of these statements will be considered later on in the paper.

The two greenish black individuals (fig. 7) with white throats and breasts resemble strongly the variety of ducks known as Blue Swedish. One died at an early age. The other shows

traces of light head stripes. She has some white primaries. The under side of her wings instead of being white are light brown with darker markings. The speculum is obscured, apparently by being overlaid with black pigment. The bill is nearly black with greenish cast. The feet contain much black in addition to the normal orange. The occurrence of similar black ducks has been noted in two other flocks composed of Pekins and Rouens.

THE F_2 GENERATION

The F_2 generation is a motley assemblage. Unfortunately many of the young managed to lose their leg bands so that the exact parentage of each individual in not known. Consequently this generation will have to be considered as a whole, except in the case of pigmented and non-pigmented down, which is given in connection with the description of each mating. The adults are considered under a separate heading.

A record of the down plumage of each duckling was made at hatching time. The only correlation between down and adult plumage, about which there is no doubt, is that existing between yellow ducklings and white adults. There are several types of down among the pigmented ducklings, but their relation to the various types of pigmented adults is entirely unknown, so that the down records only enable us to separate pigmented from non-pigmented individuals.

F_2 matings and down color. Two pens of F_1 were mated. In one a drake from a Pekin female by Rouen male was placed with two of his sisters. Their eggs did not hatch well and only nine young were produced. Six had pigmented down and three yellow down.

In the other pen was placed a drake, derived from a Rouen mother and Pekin father, with one of his sisters and the F_1 black duck with white breast. From the first duck there were five pigmented and four yellow ducklings; from the black duck there were seven pigmented and three yellow ducklings.

Altogether there were twenty-nine F_2 ducklings from these two pens, nineteen were pigmented and ten yellow. If the black

duck's progeny is left out of consideration there are twelve pigmented and seven yellow ducklings. In any case there is a considerable excess of yellow ducklings, the proportion standing about two pigmented to one yellow, instead of the expected ratio of three to one.

Later in the season, a Pekin drake was substituted for the hybrid drake in the second pen. There were seven pigmented and three yellow ducklings. Only one was reared, a male. He is not included in the table given below.

A mating was also made between a hybrid male and a Pekin female. Only three ducklings were hatched, two pigmented and one yellow.

F_2 *adults*. The chief interest in the F_2 adults attaches to the pigmented types, which numbered twenty-one individuals, thirteen females and eight males. The white adults are identical with the Pekins. The distribution among the pigmented adults of the various characters studied are given in tables 1 and 2. But there are also some points which require a more extended treatment.

Two females (fig. 7) and one male were essentially like the F_1 black female, though the male and one of the females had a small amount of white, distributed irregularly on the head and neck. This last female retained her leg band. Her mother proves to be the black F_1 female and her father a hybrid male. The black male has no white neck ring, no white primaries and no vermiculations. His is the only case in which these characters have been found absent in the male. His head, where not white, is lustrous green as in normal Rouen males.

The other eighteen individuals, show mostly Rouen characters. In one male the claret of the breast is wanting, its place being taken by iron gray (fig. 8). The speculum of this male is obscured and the under side of the wings pigmented. The rest of the males are not markedly different from the F_1 males, except that the width of the neck ring varies considerably.

Two of the females are much marked with white (fig. 9). The neck ring extends over nearly one-half the neck and reaches up the throat to the lower mandible. There is a large white patch

PLATE 1

1   Pekin male.   × about 1/10.   The female is practically the same.
2   Rouen, male.   × about 1/7.
3   Rouen, female.   × about 1/8.
4   A, striped head.   × about 2/7; B, plain head.   × about 1/3.
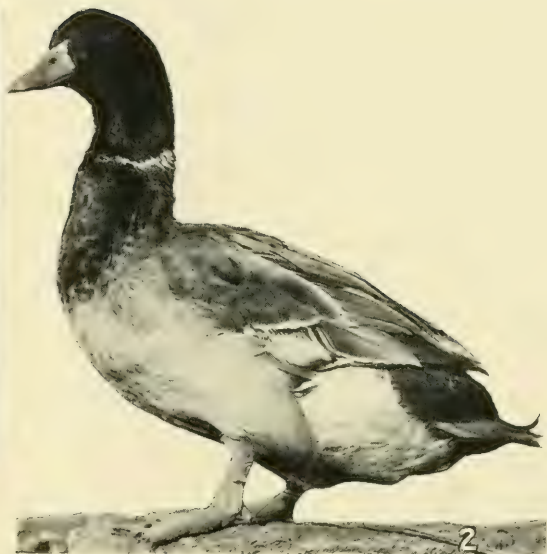
EXPLANATION OF FIGURES

5 F_1 hybrid male. $\times$ about 1/10.

6 F_1 hybrid female. $\times$ about 1/7.

7 F_2 white breasted, black female. $\times$ about 1/8. The F_1 black female is practically identical with this one.

8 F_2 male which lacks the claret breast. $\times$ about 1/7.

9 F_2 female, $\times$ about 1/9, with much white and somewhat resembling an Indian Runner duck.
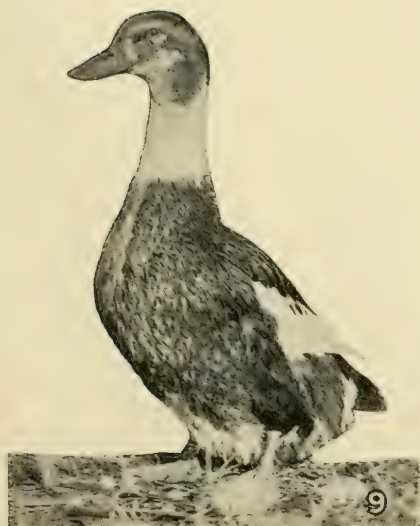
on the belly and much white in the wings. Judging from a study of the other females, there are four points or centers for the origin of these white areas, viz.: At base of lower mandible, neck ring, posterior end of sternum and outer primaries. The white areas themselves may be large or small. They may all be present or all absent, or part may be present and part absent. In the case of the two females under consideration, all are present and all large, thus producing an effect which calls to mind the pattern of Indian Runner ducks. Both these ducks have nearly yellow bills.

One female has a noticeably redder breast than the others. Its meaning is uncertain.

There appears to be a curious correlation between plain head (fig. 4B) (*i. e.*, head without stripes), obscured speculum and pigmented under surface of the wing. There are four cases of plain head among the females, not including the black individuals, two in F_1 and two in F_2. All had obscured specula and all had the under side of the wing pigmented. All the blacks have obscured specula and the under surface of the wing pigmented, though it is difficult to determine whether or not the head is really striped.

DISCUSSION

The data which I have been able to present are too meagre to permit of much discussion. There are, nevertheless, some features which are strongly suggestive.

The most striking thing about the F_1 generation is the apparently greater heterogeneity of the females as compared with the more homogeneous males. A description of one F_1 male would fit all the other F_1 males, though, of course, larger numbers might prove this statement to be without foundation. The evidence in regard to certain characters is, however, fairly strong. All F_1 males have a white neck ring and white primaries. Further, leaving out of consideration for the present the summer plumage, no case of striped head has been seen in the F_1 males. There is similar but less conclusive evidence in regard to some other characters. But there is no evidence of an opposite nature. Since in

F_1 certain characters are present in some females and absent in others, and since in the males the same characters are always present (or always absent according to the character in question) it appears that these characters are sex limited. The following generalized formula will show how the facts may be interpreted, using Bateson's assumption of a sex homozygous male and sex-heterozygous female.

S, is a sex limited character; s, its absence, *i. e.* the presence of another character. The P_1 females are S ♀ s ♂; the males S ♂ s ♂. Coupling is assumed to occur between ♀ and s. Or, if preferred, a repulsion between ♀ and S may be assumed, or a coupling between ♂ and S. The F_1 then, will be S ♂ S ♂, S ♂ s ♂; S ♂ s ♀, s ♂s ♀.

The F_1 males, then, will be visibly alike, though of two gametic constitutions; the females will be of two visible classes.

Before going further, it may be well to point out that the data at hand do not necessarily support Bateson's theory, for the facts can also be represented thus;

Females s ♀ S ♀; Males s ♀ S ♂, giving females s ♀ s ♀, s ♀ S ♀ and males S ♂ s ♀, S ♂ S ♀.

Random matings of the F_1 will give theoretical results in F_2 agreeing sufficiently well with the observed results, which ever formula is used, though according to the first method of formulation, a few males should occur without the character in question in F_2. According to the second method all the F_2 males should have the character in question. The F_2 black male lacks a ring neck and white primaries, thus rather favoring the first mode of representation. However, it will probably be necessary to establish suitable strains, before the bearing of the experiments on the present theories of sex determination becomes perfectly clear, but the experiments indicate unmistakably the existence of some sort of sex limited inheritance with respect to several of the characters studied.

In the formula given above I have assumed that both sexes of the parent stock were heterozygous for certain characters. I have made this assumption because of the variety of forms appearing in F_1 which ordinarily conforms to a single type. The unex-

pected occurrence of such characters as a *broad* neck ring in the males and the appearance of a neck ring, plain head and black individuals among the females, is probably due to the presence of cryptomeres in the Pekins, though this point cannot be proved until further crosses with other varieties of ducks are made. Ridgeway, however, mentions in passing a cross between Muscovy (black) and Aylesbury (white). The progeny were colored like mallards. His figure shows a very broad neck ring.

Thus, in many respects F_1 in the present instance, is like an F_2 generation. The F_2 generation in these experiments contains only a few more forms than F_1. Indeed, with larger numbers, some of the forms which have appeared only in F_2 may reasonably be expected to appear in F_1. A priori, it would seem more probable that the heterozygous males occurred among the Pekins. The Pekins may easily be a mixed population in respect to numerous factors rendered invisible by the white plumage. Some individuals may be heterozygous in respect to a given character while others may be homozygous either for the presence of this character or for its absence. The introduction of color has made possible the isolation of pure lines.

As the individual mothers of the F_1 generation are unknown, it is obvious that if one mother were homozygous for a character and another heterozygous, the father being homozygous, that the observed results would follow, provided that it is also assumed that in the female a character does not become patent except in the homozygous condition, though patent in its heterozygous condition in the males. Thus the case would be paralleled to the inheritance of horns in sheep, cited by Bateson.

Not all characters appear to be correlated with sex. White shading of the breast and red-white on the dorsal wing surface is present in some F_1 individuals of both sexes, absent in others, thus behaving as if it were of the form DR in both parent stocks, or DR in one and RR in the other. The evidence, however, is conclusive that at least one of the parent stocks contains individuals which are heterozygous for certain characters.

EXPERIMENTS ON ASYMMETRICAL FORMS AS AFFORDING A CLUE TO THE PROBLEM OF BILATERALITY[1]

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ELEVEN FIGURES

ONE PLATE

I

In bilaterally symmetrical animals we may often find monstrosities consisting in the appearance of a pair of supernumerary appendages, of which one shows the symmetry proper to its side, while the other, being the mirror image of the former and also of the normal appendage, shows the symmetry of the opposite side. Two photographs, the first of a bull with a supernumerary pair of legs arising from its right shoulder (fig. 1) and the second of a trifid dactylopodite in Cancer pagurus (fig. 2) will serve to illustrate the statement. Is the appearance of appendages with an inverted symmetry really due to the presence there of latent determinants of the opposite body-side, or is it merely due to development of an anlage of its own body-side, the mirror appendage standing in no causal relation whatever to the opposite side?

A perfectly symmetrical form affords no clue towards the solution of the question, because on such objects we have no means of distinguishing a structure in the proper position but developed in an inverted fashion; from a structure of the opposite side. In animals, however, where unequally developed appendages are

found on the right and left part of the body, this may well be attempted. It may be recalled that many decapod Crustacea present an unequal development of the chelae, a phenomenon which I had described as 'heterochely,' and of which the photograph of Alpheus ruber (fig. 3) may serve as an example. All crayfish in which heterochely is independent of their sex, and which show no other asymmetry in their organization, are capable of transforming the small chela into a large one, while a small chela regenerates in the place of the original large chela, if the removal of the large chela occurred at a sufficiently early stage. This reversal of chelae was first observed in Alpheus dentipes, of which a photograph is shown in fig. 4.

The interchangeability of the chelae indicates that the capacity of forming a large chela is present on both sides of the organism, but the development of the new potentiality and the transformation does not involve a reversal of the symmetry of each chela; on the contrary, while the crusher and nipper character of the claws become thus reversed, both the right and the left chelae retain the symmetry of the right or left side. Whereas in Alpheus individuals not operated upon may have the large or crusher claw either on the right or left side of the body, there exist other heterochelous decapods in which the first differentiation of the crusher claw occurs always on a definite side.

In our common heterochelous crabs, Carcinus, Portunus and Eriphia (the last is shown in fig. 5) this is always the right side, and left handed individuals, as I have proved experimentally, arise through the reversal of the claws occasioned by the loss of the right chela. When big crabs are used, representing older stages, the change from the right-handed to the left-handed condition proceeds very slowly, and at first the chelae appear of equal size, because the regeneration of the small claw instead of the large crusher is fully completed while the previously small, left claw has not yet transformed itself into a crusher. This secondary homochely is illustrated by a photograph of a regenerating Eriphia (fig. 6), which represents also a monstrosity of an extra double dactylopodite borne on the right chela. This monstrosity found in nature must be recognized as having been

produced by regeneration, because the structure of its tubercles and teeth does not correspond to that of a large crusher claw, but of the nipper claw, while the nipper on the left side has not yet been transformed into a crusher. We learn from this abnormal case that the right chela is not only capable of taking on characters of the normally small, left claw but also of developing nipper dactylopodites with the symmetry of both the right and left sides. But the secondary homochely in this case prevents us from solving the problem, which was presented at the beginning of the paper. We must look for an object, in which the heterochely remains unchanged when a supernumerary appendage with secondary symmetry has arisen. The lobster furnishes such cases (Homarus, fig. 7) because if the well developed crusher is removed no reversal takes place, but a new crusher regenerates in the old place, the nipper claw remaining unchanged. But during their regeneration the large claws pass through stages which resemble the nipper claw, having an equal, fine dentition, interrupted by enlarged teeth, while the true crusher is characterized by a rough and irregular dentition.

Monstrosities with triple chelae are not infrequently met with in the lobster and are undoubtedly due to regeneration, because such have also arisen in experiments, and furthermore because abnormal appendages, very apt to be thrown off during the successive moults, are generally found in lobsters of considerable size. In the course of the past few years I have been searching through the literature for references to such monstrosities, and have especially searched through museum-collections and have made a comparison of these triple chelae of lobsters, the results of which may be summarized thus:

1. Supernumerary appendages with secondary symmetry in the small or nipper claw exhibit always the characters of nippers and even those with the symmetry of the opposite body-side form no part of the large, or crusher-claw, as will be observed in the photographs, figs. 8 and 9.

2. Supernumerary appendages with secondary symmetry in the large, or crusher claw, have the same characters in both divi-

sions and also do not show the normal differentiation of the right
and left body-side.

3.   In monstrosities of this kind which are already well devel-
oped, both divisions of the appendage present the full character
of a crusher.[2]

4.   If, on the contrary, the monstrosities are not yet com-
pletely developed, they always show transitional stages between
the nipper and crusher claws (figs. 10 and 11.)

From these results, which are corroborated by instances from
all other heterochelous crustaceens, we may, I believe, draw the
conclusion, that monstrosities with an inverted symmetry are
not caused by the appearance of determinants of an opposite
body-side, but by the inverted growth of the anlage of the same
body-side. As we have no reason to attribute the quite analo-
gous monstrosities of appendages with inverted symmety occur-
ring in strictly bilaterally symmetrical animals to causes different
from those which are responsible for minor asymmetries, we may
therefore extend the above conclusion also to all bilaterally
symmetrical animals.

II

Now, let us turn from the province of regeneration to that of
embryology and consider the fundamental experiment of Roux
by which he was able to show that if one of the two first blasto-
meres of the frog's egg is injured, the remaining one was able to
develop alone and to produce a half-embryo representing either
the left or the right side of the organism. Thus with this expe-
riment Roux proved the possibility that the first two blastomeres
give rise respectively to the left and to the right part of the body
in typical development. Further experiments however, by Roux
and others, soon demonstrated in the case of the frog as well as
of other animals the possibility of an *atypical* development,
where from one of the first two blastomeres a complete embryo
may arise, both sides of the embryo being thus produced from

[2] A good case of this kind has been photographed recently by Leon J. Cole,
Biological Bulletin, vol. 18, 252, 1910.

a blastomere destined to produce in typical development only one side of the body. Before proceeding further I should like to point out the fact that the experimental production of two whole embryos from one egg was successful only in those cases where the first cleavage furrow separates the right and left elements of a bilaterally symmetrical animal, and not in those cases where the first cleavage divided the dorsal from the ventral half. As I have indicated already in the "Festschrift für W. Roux" the dorsal and ventral Anlage are incapable of restoring reciprocally one another. Those examples, where in the same species the first cleavage furrow may either typically or atypically take a different course, are extremely suggestive, for if a right half is separated from a left half the reciprocal restoration is possible, while dorsal or ventral halves give rise only to incomplete embryos. We may recall in this connection Spemann's interesting experiments on eggs of Triton and especially the atypical course of the first cleavage in Ascaris-eggs induced by centrifuging in experiments of Boveri and Hogue, also published in the Roux-Festschrift 1910, but not known to me at the time I arrived at the above conclusion.

Considering now the results of regeneration in the light of the results of embryology, it would seem simplest to assume that the right and left sides of the body are not at all self-differentiated, but that they are conditioned by the other body-axes. In this way the establishment of two axes each with two distinct poles (anterior and posterior; dorsal and ventral) would fully suffice to determine the bilaterality of the organism, because, if we presuppose a similar interaction of analogous anlagen, those lying in the third axis and giving rise to the right and left half of the body should naturally arrange themselves so that they would represent mirror images of each other, when they occupy the same position in relation to the two differentiated axes.

The problem of the determination of axes was also put forward with great clearness by Roux. He contrasted the differentiation of the right and left body-side, induced by the meridian of fertilization, with the predetermined and readily observable dorso-ventral differentiation of the frog's egg. Inasmuch as the

PLATE I

(For figs. 2, 8 and 9 I am indebted to C. Carlgren of Stockholm; fig. 1 was taken at a show, fig. 7 is a copy after V. Emmel, figs. 3, 4, 5, 6, 10 and 11 represent preparations in the Museum of the Biologische Versuchsangstalt, Vienna.)

1 Bull with pair of extra appendages attached to right shoulder.

2 Cancer pagurus, chela with pair of extra dactylopodites attached to base of normal dactylopodite.

3 Alpheus ruber, from below and from above, showing normal Heterochely.

4 Alpheus dentipes, several of chelae, after autotomy of left big claw, (shown above).

5 Eriphia vulgaris, normal right-handed individual (above) left-handed individual (below) both seen from below.

6 Eriphia vulgaris, from below, bearing a pair of supernumerary dactylopodites on right chela.

7 Lobster, normal crusher and nipper claws, from above.

8 Lobster-claw with pair of extra propodites arising from propodite of a nipper claw.

9 Analogous case, further developed condition, (the dactylopodite is not photographed in this case).

10 Lobster with pair of extra dactylopodites at base of the dactylopodite of crusher claw, from above.

11 Same individual from below.
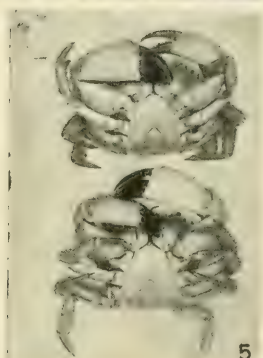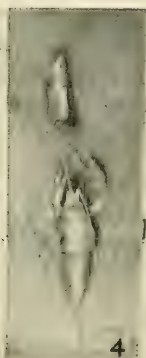
antero-posterior differentiation falls upon the meridian of fertilization we may justly suppose that the grouping of anterior and posterior anlagen is also produced through fertilization. The symmetry of the right body-side would be developed on the right of the meridian passing through the posterior and dorsal poles, while the symmetry of the left body-side would develop on the opposite side. Strictly bilaterally symmetrical animals do not afford the means of discerning as to whether similar or dissimilar "Anlagen" are distributed in both sides of the body.

III

We must come back now to the topic of minor asymmetry and to consider such objects which may be propagated through several generations, because through the study of heredity we must find out if the asymmetry, manifested on a certain side of the parents body either in the development there of a new character or in the dropping out of a character, will tend to reappear also in the offspring; and, furthermore, whether the asymmetry will reappear on the same side of the body, or on any side quite regardless of the condition observed in the parents. The phenomenon of heterochromy of the eyes presents such a case of asymmetry, as it is found for instance in certain cats with one yellow and one blue eye. From my lists of pedigrees of these cats I made the following deductions:

1, Heterochromy as such may be inherited; 2, in this case the offspring does not always have the yellow eye on the same side of the body as its parents; and 3, the color of *one* eye of the ancestor may in the offspring appear in both eyes.

Other authors, for example Castle, found it impossible to fix color-patches tending to asymmetrical distribution in descendants of guinea pigs selected for this purpose. On the contrary the symmetrical hood of piebald rats, which extends over the anterior and dorsal half of the animal is inherited as an unchangeable unit-character in strict accordance with Mendel's rule.

Apparent exceptions to Mendel's rule frequently appear in those structural characters which are often asymmetrical, as for

instance supernumerary toes of chickens, as was found by Barfurth, Bateson and others. In their experiments, too, the hyperdactyly appeared sometimes on the right, sometimes on on the left and sometimes on both sides quite independent of the parental condition. Although generally dominant, hyperdactyly may in some individuals become recessive, reappearing again in the next generation as a typical dominant. This fact is explicable according to my view, namely, that in each egg the "plus toe Anlage" is redistributed, so that it goes either to the right or to the left, or to both sides of the body, or even to parts of the body not capable of producing toes, in which case the Anlage does not become manifest. I may also add that Kammerer produced symmetrically spotted Salamandra maculosa from irregularly spotted parents, in which the yellow blotches had been enlarged by keeping them on yellow soil. We find here that the induced 'plus' of yellow in the parents, is redistributed in their young according to the bilaterality of the body.

IV

From all these facts derived from the study of regeneration, embryology and heredity, may we not draw the conclusion that there are no distinct determinants for right and left body-sides; but that the bilaterality depends entirely upon the distribution of anlagen in opposite directions with reference to the dorso-ventral and antero-posterior axes? True, it seems difficult to reconcile the conclusion with those asymmetries, which are confined not merely to minor parts, but are manifested in the structural plan of the entire organism, as for instance the asymmetrical position of the heart in vertebrates or the dextrosity of snail shells. But such asymmetries are transmitted unchanged from one generation to the other, and the question therefore arises, if for these cases we must not postulate specialized anlagen for right and left body halves, each incapable to replace the other? I believe we must not, because, even though rarely, we do nevertheless find a reversal of the fundamental asymmetry, as the "situs inversus viscerum" in vertebrates or sinistrosity in

snail-shells, in individuals belonging to a species which is normally asymmetrical in the opposite direction. To my knowledge there exist no human families with situs inversus viscerum, and Lang had no success in raising sinistral snails by inbreeding two sinistral individuals for two generations. We must, therefore, see in these instances of reversed asymmetry nothing else than somatic transpositions, such as may also be induced artificially according to Crampton, by compressing the snail-eggs. And as was already suggested by Conklin the transpositions are brought about by the inversion of the relative position of the dorso-ventral and antero-posterior anlagen. But in the germ-cells of the inverted body the normal distribution reappears, thus indicating the normal dextrosity of the dorso-ventral and antero-posterior anlage.

Closely analogous to this seem to be the minor asymmetries which have a fixed position from birth but later in life may become inverted, as for instance in the case of the dextrochelous crabs, whose fossil predecessors have already borne the big claw attached to the right side of the body.

Of the causes of these asymmetries and why in certain species *one* side of the body is particularly predisposed to modification, we know nothing, but it was not even my purpose to discuss here this matter. I merely wished to point out that the study of minor asymmetries yields to us the key to the problem of bilaterality, with which we may now attempt to unlock the doors separating us from the complete understanding of this problem.

STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

I. THE AXIAL GRADIENT IN PLANARIA DOROTOCEPHALA AS A LIMITING FACTOR IN REGULATION

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CONTENTS

INTRODUCTION

With the development of the experimental method in zoölogy an extensive literature on form regulation has arisen: a considerable portion of this literature, however, either tacitly accepts or positively maintains the view that the phenomena of regulation are distinct from those of 'normal' development and require

a special mechanism. To others it seems possible to account
for the observed facts only by the assumption of an 'entelechy'
or other vitalistic principle. Only here and there do we find a
definite and conscious attempt to show that life and regulation
are in large measure the same thing, that the mechanism of regu-
lation is the mechanism of life and that form regulation so-called
is merely morphogenesis occurring under certain special condi-
tions.

As considered more fully in another paper, soon to appear, my
own position is that the problem of regulation is, at least biolog-
ically, the problem of life. Regulation is the process of equili-
bration in the system, following a change in the medium or envi-
ronmental conditions. Without the change in conditions which
within certain limits, leads to regulation, or without the power of
equilibration, life would sooner or later cease. On the other
hand, equilibration, i. e., regulation if some sort *must* result from
changed conditions, as long as the dynamic processes are not com-
pletely inhibited or the system otherwise destroyed by the changes.

From this view it follows that in the formation of a whole from
a part artificially isolated we are concerned with processes of
reproduction and development and that here as in the formation
of a new individual from a fertilized egg, the problem of heredity
is given in its essential terms. We see from experiments on
regulation that physical or physiological isolation of a certain
degree or kind is a necessary condition for the initiation of the
change toward wholeness in a part. Moreover, many, if not all
forms of reproduction in nature differ from our crude operative
isolations chiefly in that the isolation of the part which forms the
reproductive element is brought about by physiological condi-
tions in the organism rather than by mechanical means (Child,
'11a)

In the reconstitution of the whole from a part all the essential
features of the reproduction of an organism of specific character
from a reproductive element of a certain constitution are as truly
present as in the development of the egg, though in different
form because of the difference in conditions. Whether we call
the process regeneration, restitution or reconstitution, whether

it involves extensive redifferentiation in the isolated part or is chiefly limited to localized outgrowth and differentiation of new tissue, it is development, morphogenesis, just as certainly as is the formation of an organism from the egg.

Furthermore, wherever a new whole, a new 'individual' arises in the organic world, there we have before us the problem of heredity. But we must pause here for a moment to consider what we mean by heredity and inheritance. To say that inheritance is resemblance between offspring and parent or between related individuals is incorrect, for such resemblance may arise from external conditions acting during development, as well as from something which the reproductive element brought from the past to the beginning of development. To assert that inheritance is the transmission of characters from parent to offspring is also unwarranted, since we know nothing of 'characters' except as they appear in the structure and behavior of the organism during and after ontogeny, and we know but little of transmission or its possibilities.

Godlewski ('09) has recently given the following definition; "Vererbung ist die Fähigkeit des Organismus den morphologischen Ausgangspunkt seiner Entwicklung aus einem bestimmten Teil seines eigenen Korpers auszubilden und vermittelst desselben seine Eigenschaften auf die Nachkommenschaft die sich daraus entwickeln kann, zu übertragen." This definition does not seem to me to cover the ground exactly or fully; how do we know that the organism differentiates or develops a certain part as the morphological starting point of its development? For it to accomplish this a certain degree of finality would seem to be necessary. And again, we do not know that the organism 'transmits' (übertragt) its characters to anything, indeed there are strong reasons for believing that it does not.

A definition of heredity which is broad enough to include all kinds of heredity and which does not involve premature and unwarranted conclusions as to the nature of the process of inheritance, must, it seems to me be somewhat as follows: *Heredity is the sum total of the inherent capacities or 'potences' with which a reproductive element of any kind, natural or artificial, sexual or*

asexual, giving rise to a whole or to a part, enters upon the develop-mental process. And the process of inheritance is the process of origin and development of these capacities in the reproductive element. In short, heredity is what the reproductive element brings with it from its past. If the sex cells, like the asexual reproductive elements and the artificial elements resulting from the isolation of pieces by operation, are at some earlier stage of their development physiologically parts of the organism, and there are very strong reasons for believing that they are, then we may define heredity as *the capacity of a physiologically or physi-cally isolated part for regulation.*

The only way in which we can discover anything concerning this capacity is by allowing the regulation to occur under the most various and carefully controlled conditions, *i. e.*, we can investigate and analyze the problem of heredity only with the aid of development. At some time in the future it may be pos-sible to determine directly by physical and chemical analysis of the reproductive element what its capacities for development are, but at present we know so little concerning the relation between constitution and capacity that for the most part we can recognize capacities only as they are realized in the course of development.

Heredity is involved not only in the development of new wholes from reproductive elements but in the reproduction of parts. Every cell division is as truly a special problem in heredity as is any other reproduction. The reappearance of the same number of chromosomes in successive generations of cells is as truly a case of heredity as is the reappearance of five fingers on the hand in successive generations of man. Montgomery has said that we understand heredity so far as we know the behavior of chromo-somes (Montgomery, '06, p. 56). It seems to me that it would be much more nearly correct to invert this statement and say that we understand the behavior of chromosomes so far as we understand heredity.

As soon as we view the problem of heredity from this broad general standpoint it becomes clear that wherever in the organic world reproduction occurs, there is heredity also. Moreover,

it also becomes clear that in the products of sexual reproduction in the higher animals heredity appears in its most complex form and under conditions which render analysis almost impossible. Doubtless we can establish rules which will have a greater or less empirical value, but the conditions of sexual reproduction are such as to make the problem one of the most if not the most difficult and inaccessible with which the biologist is concerned.

In the simpler forms of asexual reproduction in the simpler organisms, there exists the possibility of a greater degree of control of the conditions of reproduction and development, and therefore of further insight into the nature of inheritance. And finally in the experimental reproductions, i e., the processes of reconstitutional regulation following the physiological or physical isolation of parts of the organism, there exist still further possibilities of control and analysis, which are not present in either asexual or sexual reproduction in nature. I believe there is much to be learned from these simpler forms of reproduction that the breeding and crossing of sexual forms can never teach us: I believe further that our hypotheses and theories of heredity must sooner or later take as their starting point the simplest phenomena of reproduction and development, not the most complex.

I am well aware that objections to this point of view may be raised. It will doubtless be maintained by some that inheritance from pieces of the soma is quite a different matter from inheritance through the germ plasm. Such objections are based merely on theoretical grounds and my reply is simply that 'germ plasm' exists wherever reproduction occurs. I believe we are logically bound to extend the conception of heredity to every form of reproduction. Moreover, there is much evidence, which I hope to discuss at another time, in support of the view that the sex cells are primarily just as much a physiological part of the individual organism as other organs and that they undergo sooner or later in the course of their differentiation a process of physiological isolation. Some of this evidence I have presented briefly in other papers (Child, '11a, pp. 81–88 '11b). In short there are no facts which contradict, and there are many which

support the conclusion that inheritance is fundamentally the same problem wherever a new living system arises from a part of a preëxisting system.

The presentation of the following experiments on form regulation as in part a study on heredity is then merely the logical consequence of a conception of heredity which I believe must sooner or later find general adoption.

The further objection may be made that in the development of isolated pieces from adult organisms so many secondary factors connected with the morphological differentiation are involved that the prospect of any real advance in our knowledge along these lines is but slight. To such objections we may reply that morphological differentiation exists in every egg cell, and moreover, the fact that neither the egg in most cases, nor the spermatozoön, is capable of initiating its own development when isolated, indicates that before their union both these cells are, at least often, more highly specified physiologically than, for example, portions of a hydrozoön or planarian body, in which physical or physiological isolation alone, without any stimulus comparable to fertilization, is sufficient to initiate the development of a new whole. The idea that the simplest, most primary conditions of development exist in the gametes or the zygote is, I believe, very far from being correct. The gamete before maturation is very evidently a highly differentiated cell, it is physiologically aged and in some cases in the last stages of senility and approaching death (Child, '11b). Moreover, sexual reproduction is a form of reproduction characteristic of relatively old organisms; it is the last form of reproduction in the reproductive cycle. To regard this as the most 'typical' or the most important biologically of the different forms of reproduction, and the one on which alone we should base our theories or heredity, is to close our eyes to a large part of the most significant facts of organic life.

In the course of these studies I shall indicate what I consider to be the bearing of the facts upon the general problem of heredity, as well as their relation to the more special problems of heredity which are associated with the particular form of reproduction involved. The work already accomplished during the

last ten years and to be presented in this and several following papers is merely preliminary to more extended investigation along various lines, some of which is going on at the present time.

Before turning to the experimental data it is necessary to point out their bearing upon certain questions more directly connected with the special field of form regulation. It is an interesting fact that in spite of the extensive literature on the phenomena of form regulation in Planaria many features which demonstrate the close relation between the processes and results on the one hand and various factors, internal and external on the other, have received little or no attention. No species of Planaria which has thus far been subjected to experiment is at any given time in its development actually an equipotential system, yet the apparent equipotentiality of parts seems often to be regarded as the chief result of earlier work upon this genus. In the interest aroused by the remarkable capacity of planarians as well as various other simple forms for reconstitution of wholes from parts, but little attention has been paid to the factors which limit this capacity. If our investigation and analysis of such factors had been more extensive and rigid in the past it would never have been possible to regard either Planaria or other simple organisms as equipotential systems. As a matter of fact the limiting and determining factors are perhaps of even greater interest and importance than the great capacity for regulation, for it is these factors which enable us to learn something of the character and relations of the processes involved. The existence of such limits of regulation is totally ignored by Driesch in his definition of the equipotential system. It is certainly not true, either for Planaria or for any other form that any part is capable of producing any other part, or that any part is capable of producing a whole. Only pieces of a certain character possess such capacities, and we are already able to determine some of the factors which go to make up that character.

Moreover, as will appear below, a 'whole' is not something with clearly defined characteristics, but is rather a very vague notion. Actually our brief examinations of the results of form regulation are totally inadequate to determine whether they are 'wholes'

or not.   We can only say that they seem to be wholes.   But we
can see very clearly that such wholes are not alike and we shall
find that in Planaria their unlikeness is, at least in certain respects,
not a matter of 'chance' but is, subject to certain laws.   The
point where 'wholeness' ceases to exist is not a definite point
determined in nature, but an arbitrary convention of the human
mind.   Cases of resemblance to the original animal or to the
'norm' within certain limits we distinguish as wholes from cases
where the resemblance is less.   In short we have here a distinc-
tion of the same kind as that existing between 'normal' and
'abnormal.'   The normal is merely the usual under the usual
conditions.   It is not at all difficult to distinguish between a
'normal' or a 'whole' planarian on the one hand and a tailless
head or a headless tail on the other, but between these different
extreme forms we find many intermediate gradations and doubt-
less many others exist which we cannot distinguish.   We cannot
determine where the organism begins to become headless or tail-
less or in general abnormal, because this point is a human con-
vention not a datum of nature.

And finally attention may be called to the fact that the experi-
ments on form regulation afford facts and conclusions of great
interest for the general problem of physiological correlation: they
enable us to learn more of the physiological character of organic
individuality than is at present possible in any other way.

The present paper is concerned primarily with the demonstra-
tion of, first, the existence of certain differences in constitution
along the chief axis of the planarian body, and second, the exist-
ence of physiologic correlation between these regions of differ-
ent constitution.   The method of investigation is the compari-
son of pieces of the same size from different regions and of pieces
of different size from as nearly as possible the same regions.
What I have termed the constitutional and correlative factors
in the regulation of pieces are in other words the factors of size
(length) of the piece and region of the body from which it is taken.

EXPERIMENTAL DATA

1. Material and methods

My experimental work on the genus Planaria has extended over a period of more than ten years: during almost every year of this time at least a month or more has been devoted to this work and since 1907 work on Planaria has been going on during at least eight months of the year. Planaria dorotocephala has served as the subject of experiment to a greater extent than any other species, though a considerable amount of work has been done on P. maculata and some on P. simplicissima and on a species to which C. E. Stringer ('09) has recently given the name Planaria velata. I have also been able to extend my experiments along certain lines to Phagocata and Dendrocoelum and the marine Procerodes. P. dorotocephala and P. maculata are very similar in their general constitution, both reproduce by fission and both show much the same regulatory capacity. The formation of new zoöids at the posterior ends of these animals introduces certain complications into the results of experiments, but when once the presence of a second zoöid as a physiological, if not a visible morphological system is recognized, these complications are not difficult to interpret. My conclusions are based largely upon the study of P. dorotocephala and P. maculata. I shall refer to the other genera and species only so far as they possess a special interest in certain connections.

In experiments concerned, as many of mine have been, with the factors of size of the piece and the regions of the body, it is necessary to select the individuals for experiment first with respect to uniformity of size, for differences in size usually mean differences in age or nutritive condition: second with respect to the length of time which has elapsed since the preceding fission. In animals which have undergone fission very recently the anterior individuals still show the lighter-colored new tissue at the posterior end and are less slender than others, while the posterior individuals are of course small and possess new heads. The anterior products of fission, however, can for a long time be dis-

tinguished from animals nearly ready for fission by the difference in position of the pharynx. In animals approaching fission the postpharyngeal region is longer than the prepharyngeal, while for a considerable period after fission the reverse is the case in the anterior product. In the large individuals the growth is chiefly in the postpharyngeal region, more specifically in the second zoöid, consequently after a certain length is attained further increase in length is almost wholly postpharyngeal. After fission the postpharyngeal region of the anterior product gradually elongates again. By selecting individuals of a certain length in which the position of the pharynx is approximately the same it is possible to attain a certain degree of uniformity as regards the physiological condition in respect to fission.

Furthermore, uniformity with respect to nutritive conditions must also be considered. Starved animals give different results from those which are well fed. In a stock collected at one time from a single locality or kept in captivity and fed at regular intervals, the individuals of a certain length may be regarded as uniform in nutritive condition within certain limits. As I have shown elsewhere (Child, '11b), the nutritive reserves in well fed individuals of Planaria probably last for some three or four weeks under average conditions, i. e., the animals can go for three weeks or more without food before they begin to exhibit the symptoms of extreme starvation and rapid decrease in size. Animals left for a week or two without food show no very great differences in their regulatory capacity from those fed daily up to the beginning of the experiment.

And finally, since temperature and oxygen supply influence the course and results of regulation, uniformity or control of these external conditions during experiment is also necessary if results which are really comparable are to be obtained.

For all the series described in the following section and for others, which, together with these, form the basis of my work on the factors of size and position, well nourished individuals were selected averaging about 15 mm. in length in some cases 12–15 mm., in others 14–18 mm., and with the postpharyngeal region longer than the prepharyngeal.

In the study of the factors of size and region two methods of section were chiefly employed. One of these, most used in earlier experiments, consists of simply cutting the body, beginning at one end, into successive pieces as nearly as possible equal in length, and recording the result for each piece. It is impossible, however, with this method to avoid very considerable differences in size of different pieces, consequently in later experiments a somewhat different method was employed which gives much more satisfactory results as regards uniformity of length. This method consists in cutting the body into halves or thirds and then halving each of the pieces thus obtained and the resulting pieces again or even a third time if desirable. It is much less difficult to divide a given piece into approximately equal parts than it is to cut successive pieces of the same length from one end of an animal.

In a large number of my experiments each individual piece was isolated in a dish and a separate record kept for it. These experiments were of great value for they enabled me to distinguish certain differences in individuals which would scarcely have been noted otherwise. But after some of the factors which determined these differences were recognized and it became possible to select with a fair degree of accuracy worms which were in the same or nearly the same physiological condition, it became desirable to obtain more general expressions for the differences correlated with differences in length of piece and region of body. To obtain such results each of a number of worms of the same size and as nearly as possible in the same condition was cut into the same number of pieces and all corresponding pieces were placed together. In such series a record was made for each piece. but there was nothing to show which pieces in two different sets belonged to the same individual worm. This method has the advantage, however, that it permits compact and relatively exact expression of the results, as the tables and curves will show. The data considered in the present paper are all obtained by this second method of experiment, though many other 'individual series' might have been added, since they give the same results, except for the greater irregularity consequent upon the method of operation.

In all such series of pieces the factors of length of piece and
region of body from which it is taken are both involved, but it
is possible to separate them more or less completely by comparing
on the one hand pieces of the same size from different regions
of the body, and on the other, pieces of different size from as
nearly as possible corresponding regions.   The present paper is
devoted chiefly to experiments of this character.   In following
papers the effect of various external and internal factors in modi-
fying the course and result of regulation will be considered.
This whole series of experiments is preliminary to further inves-
tigation and a full statement of results is desirable at this time
both because the results are of interest in themselves as a contri-
bution to our knowledge of regulation, and also because they,
or some of them possess, as I believe, a certain general and theo-
retical significance.

2.   The chief differences in the course and results of regulation
of pieces

As the first step in our consideration it is necessary to describe
briefly the general course of regulation, its variations and their
limits and the character of the results for pieces of different
size and position.   We already possess some data upon this point
from the work of Morgan and others, and I have considered the
subject briefly in an earlier paper (Child, '06b).   It will be evi-
dent, from this and the following sections that Morgan failed
entirely to understand the significance of certain of the differ-
ences in regulation which have to do with the size of the pieces
and the region of the body from which they are taken.
In the study of thousands of pieces of different size and from
different regions of the body I have found that they fall most
naturally as regards the course and results of regulation into
six groups or categories.   I refer here of course merely to com-
pletely isolated pieces including the whole width of the body,
and not to the various types of double and multiaxial forms
which result from various methods of partial isolation, unilateral
isolation, etc.   All of these groups have been observed and men-

tioned by earlier investigators, but no one thus far has analyzed
at all fully the conditions which determine their occurrence.
In the following brief description the names adopted serve merely
as convenient means of distinction.

'*Normal wholes.*' These are individuals which possess all
the visible essential parts of the animal as it occurs in nature
viz., elongated body, head with pointed auricles and with two
distinct eye spots symmetrically placed, a mouth and pharynx
and intestine with one median anterior and two lateral posterior
main branches. But such normal wholes may differ widely from
each other as regards the course of the regulatory processes
which give rise to them and also in shape, proportions, size of
head, position of pharynx, intestinal branching, etc. For exam-
ple, such a whole may possess a relatively very large head, with
new tissue extending back to the level of the auricles, a body
tapering posteriorly with a slender posterior outgrowth of new
tissue and with the pharynx near the posterior end (fig. 1). Or
it may, as in fig. 2, approach the shape and proportions of the
animal in nature. Here the head is relatively smaller than in
fig. 1, the anterior new tissue may end anterior to the eyes and
the pharynx lies near the middle of the body. Or finally the nor-
mal whole may show the form of fig. 3, with a small head and
with the anterior new tissue extending far posterior to the eyes.
In such cases the pharynx usually lies in the middle or anterior
to it.

In figs. 1 and 3 we see two more or less opposed methods of
formation of a whole. In the one (fig. 1) the anterior region is
the larger and morphogenesis goes on more rapidly in it, while
the posterior end is smaller and develops more slowly: in the other
(fig. 3) the anterior region is relatively small and its development
is relatively slow as compared with that of the posterior region.
The differences between the two methods are also clearly shown
by the manner in which the change in shape and the elongation
of the pieces occurs. In the cases like fig. 1 elongation is rapid
and the posterior region becomes very slender because the head
develops early and the animal is very active, moves rapidly and
uses only the extreme posterior end for attachment. In the pieces

like fig. 3, on the other hand, the head develops slowly and remains of relatively small size, the movements of the piece are much less rapid and the margins of the posterior half or more of the body are used for attachment. Consequently the elongation and decrease in width of the body occurs most rapidly in the anterior region. In cases like fig. 2, which are intermediate in character between the extremes of figs. 1 and 3 the processes of elongation and change of shape show, like the other processes, a course intermediate between the extremes.

From these normal wholes we find the character of the pieces diverging in two directions, first toward headlessness, the whole—headless series; and second toward taillessness the whole—tailless series.

Wholes with abnormal eyes 'Teratopthalmic wholes.' These are intermediate between normal wholes and headless forms The frequent occurrence of abnormalities of the eye spots in the regulation of pieces of Planaria has been noted by various authors, but the conditions of their appearance have not been determined. I have distinguished these wholes with abnormal eyes as a separate group because, as will appear below, they stand between the normal wholes and the third group and because their appearance can be controlled experimentally to a large extent. The teratopthalmic wholes usually do not differ essentially as regards the changes of shape and proportions, the relations of new and old tissues etc. from the normal wholes, except that abnormal eyes are of much less frequent occurrence in pieces which develop large heads like fig. 1 than in others, though even in such pieces their frequency may be altered by various external factors and so experimentally controlled. In general it may be said that the larger the head produced by the piece, the greater the frequency of normal eyes and vice versa. Many teratopthalmic wholes differ visibly from normal wholes only as regards the eyes (fig. 4), but in extreme cases the head is very small and develops very slowly (fig. 5).

The nature of the abnormalities differs widely in different cases: Fig. 6 shows a few characteristic cases. Abnormalities of position and size are shown in fig. 6, *a*, *b*, *c*; abnormalities of number in *d*, *e*,
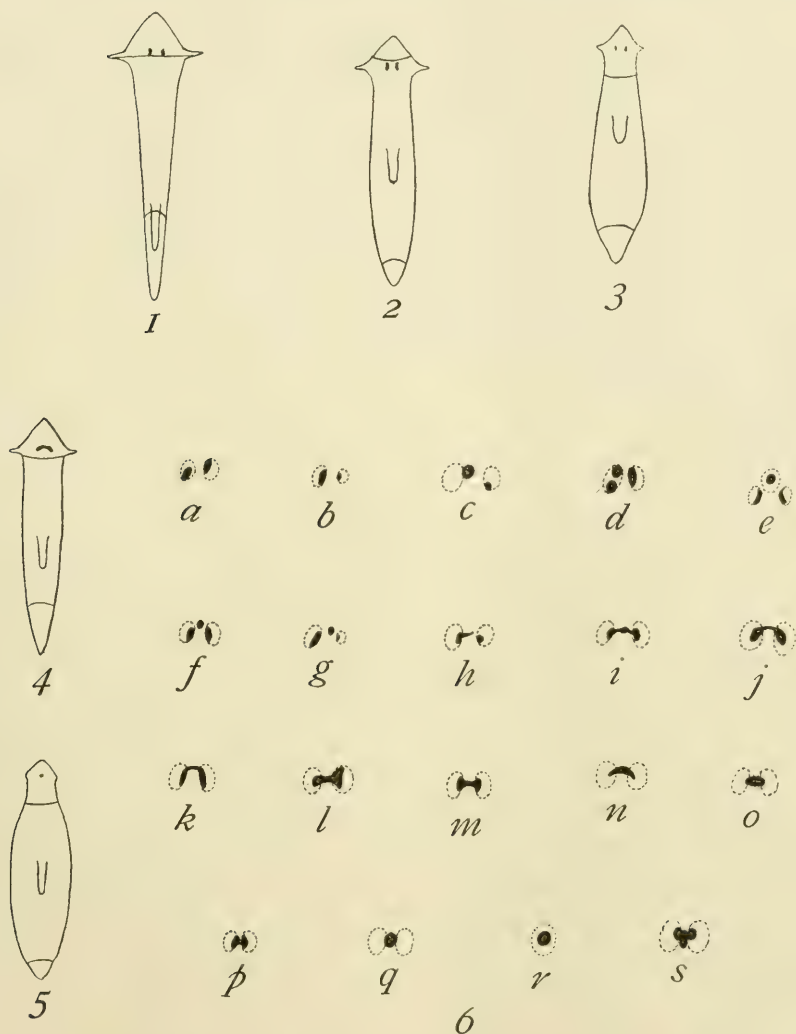
Figs. 1–3 'Normal wholes,' showing the different methods of regulation.   Fig. 1, from a piece near the anterior end: fig. 2, from the second zoöid; fig. 3 from the anterior half of the postpharyngeal region.

Figs. 4–6 'Teratopthalmic wholes.'   Fig. 4 differs from the normal whole only as regards the eyes.   Fig. 5 shows an extreme type of teratopthalmic whole with small and slowly developing head.   Fig. 6 shows various forms of 'abnormal, eyes, of which $h = s$ are the more common types.

f, g; of various degrees and kinds of union or incomplete separation in h–s. These unions or connections of the eye spots are the most frequent of the eye abnormalities and find their extreme type in the single eye spot situated in the median line (fig. 5, fig. 6, r). Differences of position and size of the pigment spots (fig. 6, a, b, c,) are not uncommon but, as noted by earlier observers, are sometimes, though by no means always, merely a consequence of an oblique cut or of other incidental conditions which delay the development of one side of the head, as compared with the other. The development of more than two pigment spots or complete eyes is of rather rare occurrence under the usual environmental conditions, but as will appear in a later paper, it can be induced experimentally. In general, the characteristic abnormality of the eyes which indicates the first visible approach toward incompleteness in head formation is the formation of eye spots nearer together than in normal animals: between this condition and forms with a single median eye all gradations exist. The various degrees in this change from the paired symmetrically placed eye spots are in general parallel to the decreasing capacity of the piece for head formation. In this and later papers various factors which influence the development of the eye spots will be considered.

'*Headless tails.*' The failure of certain pieces of Planaria to reproduce heads has been noted by various observers, but so far as I am aware, no one has stated correctly the facts concerning their occurrence. Morgan's interpretation of the absence of a head in these pieces as the consequence of the method of closure (Morgan, '98, p. 380) is incorrect, as I shall show. The head does not fail to develop because the wound closes in a certain way, but the wound closes in a certain way because the head fails to develop. The amount of new tissue formed at the anterior end varies greatly in these headless pieces: the anterior wound may remain widely open and the earlier stages of regeneration may be identical in appearance with those in pieces where a head develops. Such a case is shown in fig. 7. On the other hand, the anterior end of a headless piece may appear as in fig. 8, *i. e.*, the edges of the wound may be closely approximated and but little new tissue formed. But the analytic study of these pieces shows

clearly that the absence of new tissue is not the result of the method
of closure, but that the closure follows the absence of regulatory
growth. If the headless pieces were always of this kind, Morgan's
conclusions might be regarded as plausible, but the frequent oc-
currence of such cases as fig. 7 demonstrate its insufficiency. As a
matter of fact both size and region of the body are concerned in
these differences in the headless pieces. Of two pieces with ante-
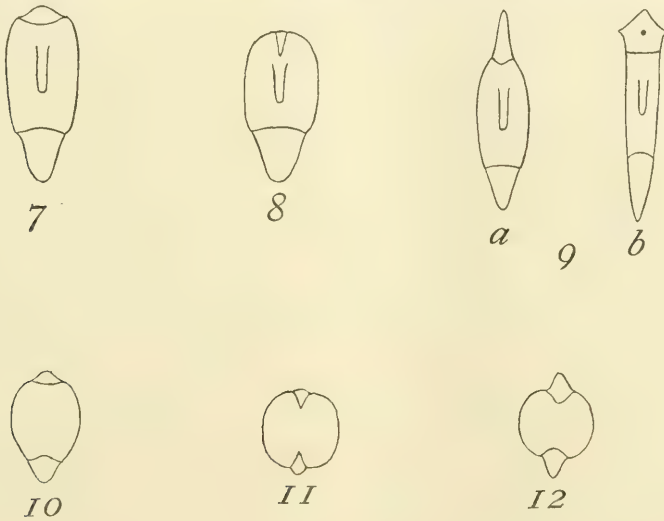
Figs. 7–12 'Headless tails.' (figs. 7–11) and 'biaxial tails. (fig. 12). In fig.
9a is shown a form intermediate between the whole and the headless tail: occa-
sionally such pieces develop a head later, as shown in fig. 9b.

rior ends at the same level of the body the larger resembles fig. 7,
the smaller fig. 8, assuming of course that both are so small that
they do not produce heads: moreover, in pieces of equal size those
further anterior in the middle half or third of the body resemble
fig. 7, those further posterior fig. 8.[1]

[1]Morgan has observed that after a second removal of the anterior end such
headless pieces produce a head, and he regards this fact as supporting his view
that the failure of a head to develop after the first operation is due to the method
of closure. In a later paper I shall show that the method of closure has nothing
to do with the result in these cases. The factors which determine whether or not
these pieces shall form a head are to be found in the constitution of the pieces and
the physiological correlation of their parts.

Between the headless pieces and the wholes various intermediate forms occur. The most common type of these is shown in fig. 9a, where a long slender outgrowth arises after a time from the anterior end, which at first was like that in fig. 7: this outgrowth is highly mobile and is used by the animal in much the same manner as a head. In most cases of this sort which I have observed development proceeded no farther than this, even though the pieces were kept for several months, but in a few cases a single median eye finally appeared in the outgrowth and in one case the elongated outgrowth underwent, in the course of several weeks, a gradual transformation into a head with a single median eye (fig. 9b). In short, the development of new tissue at the anterior end may show any condition between that of fig. 8 and a complete head and it is possible to determine experimentally with considerable exactness what stage or condition a given piece shall attain.

In certain of my experiments it has been found desirable to distinguish pieces which show a distinct anterior outgrowth of new tissue (fig. 7, 9a) from those in which the new tissue simply closes the wound (fig. 8). Various facts show that the outgrowth represents an approach to head-formation. Such pieces are distinguished as 'anopthalmic' from the strictly headless pieces.

The larger or more vigorous headless pieces always possess a well developed posterior outgrowth (figs. 7, 8, 9a), but with decreasing size and vigor the amount of new tissue produced at the posterior end decreases until it becomes impossible in some cases except when a pharynx is present, to distinguish the anterior from the posterior end (figs. 10 and 11). Such pieces are often "headless" to such a degree that not merely the head, but the whole prepharyngeal and pharyngeal regions are absent. It is impossible to draw any sharp line as regards appearance between such pieces and the fourth group.

'*Biaxial tails*'. In such pieces a posterior end arises at the original anterior as well as at the posterior end of the piece (fig. 12). Such pieces can be recognized with more or less certainty by their movements and in consequence of these movements they undergo some remarkable changes of shape, which will be considered later. In P. dorotocephala these double tails

have been observed only rarely and in very small pieces, *i. e.*, in pieces smaller than those which develop as headless. These forms are the last term of the whole—headless series: we turn now to the whole—tailless series.

'*Tailless heads.*' Just as headless tails occur under certain conditions, so do tailless heads occur under certain other conditions. Moreover, like the headless pieces these exhibit the most various degrees of 'taillessness,' from the condition of fig. 13 with wide open posterior wound and a mass of new tissue differing visibly in no way from the earlier stages of tail formation, but never developing further and never functioning like a tail, to the condition shown in fig. 14 where the wound closes almost

Figs. 13–16   'Tailless heads.' (figs. 13 and 14) and "biaxial heads." (figs. 15 and 16).

without any development of new tissue. Here, as in the headless pieces, the absence of the tail is not due to the method of closure of the wound, but the closure of the wound is the result of the more or less complete absence of growth at this end.

'*Biaxial heads*'. As the headless pieces give place with decreasing size and under certain other conditions to biaxial tails, so the tailless pieces give place to biaxial heads (figs. 15 and 16). These may be merely heads alone, as in fig. 16, or they may include the prepharyngeal regions more or less completely (fig. 15) and in some cases one or both may possess a pharynx. Not infrequently they develop a common tail in later stages from the lateral region midway between the two ends.

Cases falling under each of these six different heads have been described by other investigators, but the relation between the frequency of their occurrence and various internal and external factors has never been clearly shown. In the present paper we

are concerned primarily with certain internal conditions which
determine and limit the development of wholes from pieces,
and as we shall see, only the uniaxial forms, *i. e.*, groups 1, 2, 3,
and 5, enter into this consideration.

3.   The course and results of regulation in relation to the size of the piece and the region of the body represented

We can obtain some idea of the relation of the factors of size
of piece and region of body to the course and results of regulation
by comparison of pieces of different size and from different regions
of the body.   I have found the most satisfactory method to be
the comparison of series in which the whole body posterior to
the head was cut into two, four, six, eight, etc., pieces of as nearly
as possible equal length.   When we compare the pieces of the same
length from different regions of the body of the same or of different
individuals in similar physiological condition, the influence of
the factor or region appears, while comparison of pieces of differ-
ent length from corresponding regions shows the influence of
the size factor.[2]   The results of such comparison are briefly stated
in descriptive form with figures in the following paragraphs.

Halves.   Fig. 17, *ai* and *iq*.   Fig. 18 and 19.   Anterior and
posterior halves show but little difference in regulation.   From the
earliest stages, in which the head is distinguishable as such, up to
at least the very late stages, the anterior half (fig. 18) possesses a
somewhat larger head than the posterior half (fig. 19), and the
change in shape and increase in length are also somewhat more rapid
in the anterior than in the posterior half.   The anterior half of
large worms usually contains the pharynx, which at first lies pos-
terior to the middle, but gradually approaches it, as other
authors have noted, while in the posterior half a new pharynx
develops anterior to the middle.

[2]Everywhere except in the extreme posterior region of the body pieces of equal
length and the full width of the body are approximately equal in size.   Toward the
posterior end where the body becomes narrower, pieces of the same length may
have very different volumes and may contain very different numbers of cells.   As
a matter of fact, however, other factors render this difference practically negli-
gible in the species of Planaria which we are considering at present.
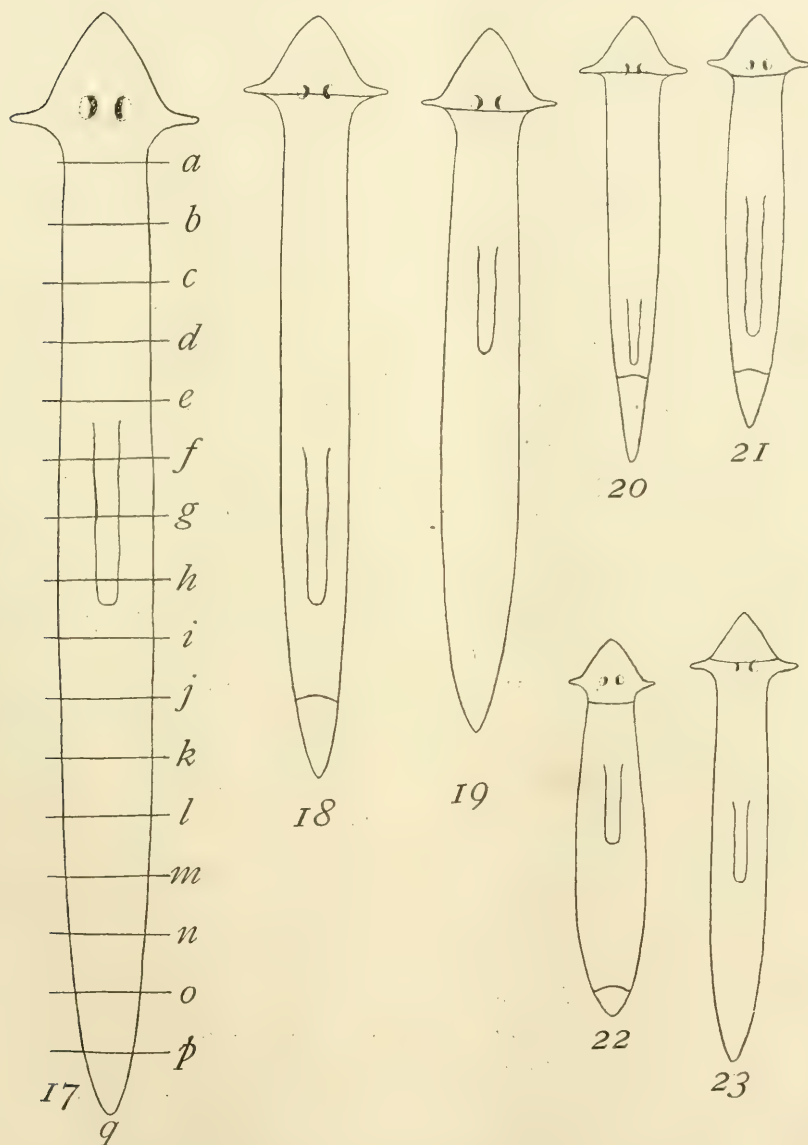
Fig. 17   Diagram indicating section of body into sixteenth pieces.
   Figs. 18-23   Fig. 18, anterior; fig. 19, posterior half; fig. 20, first quarter; fig. 21, second quarter; fig. 22, third quarter; fig. 23, fourth quarter.

Quarters. Fig. 17, *ae, ei, im, mq.* figs. 20–23. The head is largest in the anterior one fourth and decreases to the third (figs. 20–22), while in the posterior fourth it is again large (fig. 23). In well nourished individuals of good size, kept in clean water at medium temperatures the eyes are normal in the first, almost always normal in the second and third, and normal in the posterior fourth. In the anterior fourth the eyes lie just within the anterior new tissue (fig. 20), or on the line between the new and old, in the second fourth the eyes are clearly some distance anterior to the boundary between new and old tissue (fig. 21) and in the third they are still further anterior (fig. 22), while in the posterior fourth they usually lie just within the old tissue (fig. 23). In other words, the portion of the anterior end which is formed by the outgrowth of new tissue increases from the anterior end of the body posteriorly and then decreases again.

The length of the posterior regenerate is greatest in the first, less in the second and least in the third fourth. The new pharynx arises posterior to the middle in the first, anterior to the middle in the third and at the middle in the posterior fourth.

And finally the process of elongation and change of shape differs in the different pieces. After the change in shape is well advanced we find that in the anterior fourth, the body is widest just posterior to the head (fig. 20), in the second fourth it is widest at the middle (fig. 21), in the third the greatest width is posterior to the middle (fig. 22) and in the posterior fourth the shape rapidly approaches that of small animals in nature (fig. 23). In an earlier paper (Child, '06a) I have discussed the significance of the differences in position of the pharynx, amount of regeneration and change of shape in different pieces of the planarian body and the subject needs no further consideration here.

Eighths. Fig. 17, *ac, ce, eg, gi, ik, km, mo, oq.* Of the eighths I have figured only alternate ones, the first (fig. 24), the third (figs. 25 and 26), the fifth (fig. 27) and the seventh (fig. 28).

The first eighth (fig. 24) is usually a normal whole with large head with body tapering posteriorly, with long posterior regenerate and with pharynx far posterior to the middle. The second eighth is intermediate in character between the first and the third.
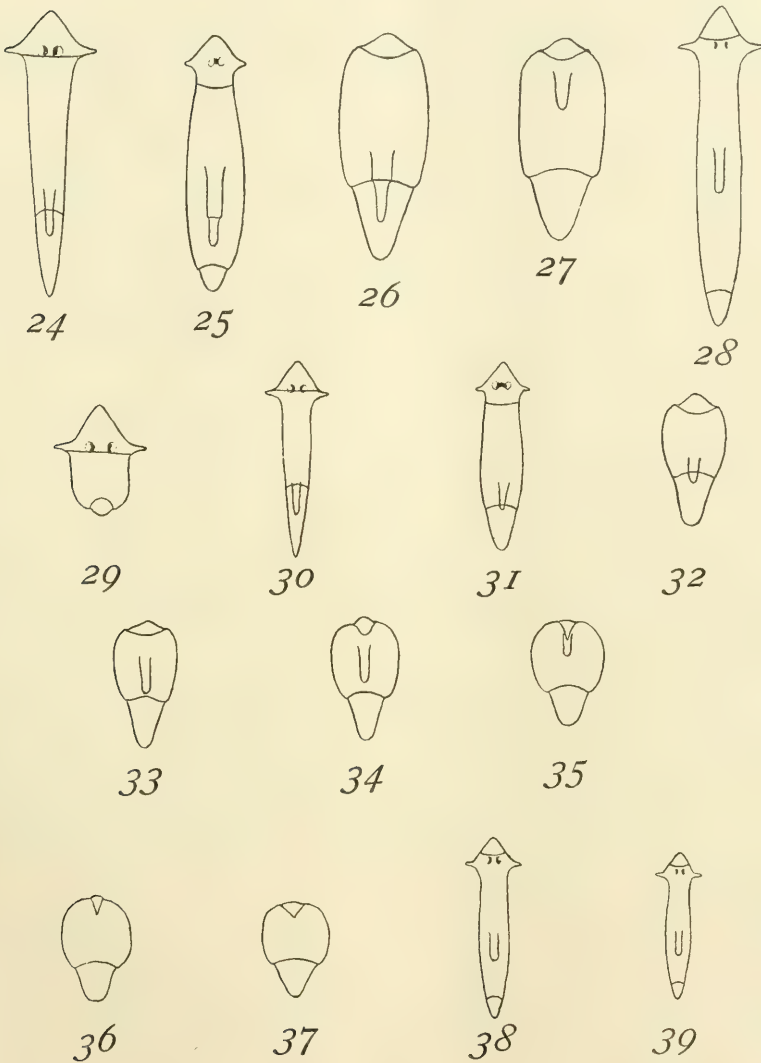
Figs. 24–28   Eighth pieces, fig. 24, first; figs. 25 and 26 third; fig. 27 fifth and fig. 28 seventh eighth.

Figs. 29–39   Sixteenth pieces.   The first sixteenth ranges between the extremes of figs. 29 and 30: the second sixteenth is like fig. 30 or 31: the third and fourth sixteenths range from fig. 30 to fig. 32: the fifth to the eleventh sixteenth pieces are like figs. 33–36: the twelfth to the fourteenth range from fig. 37 to fig. 38: the fifteenth and sixteenth are like figs. 38 and 39.

The third eighth varies in character: it may be a teratopthalmic whole of the character of fig. 25, or very rarely it may be a normal whole, but commonly it is headless like fig. 26, usually with an anterior regenerate of considerable size, but without any of the characteristics of a head. The posterior regenerate is also large. Such headless pieces undergo change in shape much less rapidly than wholes: figs. 24, 25 and 26, for example, represent pieces after the same length of time since isolation.

The fourth and fifth eighths are practically always headless (fig. 27) and the size of the anterior regenerate usually decreases from the third to the fifth. In the fifth eighth the new pharynx is anterior to the middle.(fig. 27)

The seventh eighth (fig. 28) is, on the other hand usually a normal whole with anterior regenerate ending anterior to the eyes and pharynx in the middle of the body. The sixth eighth is most commonly headless like the fifth, but sometimes forms a teratopthalmic whole, or more rarely a normal whole like the seventh. The most posterior eighth almost always forms a normal whole like the seventh (fig. 28), rarely it is teratopthalmic but never headless.

In these eighths we see the results of regulation running from normal wholes at the anterior end to headless forms in the middle region and then in the posterior fourth suddenly becoming normal wholes again. The differences in size of head, shape of body, amount of regeneration and position of the pharynx are similar to those in the fourths, but more extreme.

Sixteenths. Fig. 17, *ab, bc, cd, de*, etc. The first sixteenth after removal of the head is commonly tailless with relatively very large head (fig. 29), though it may sometimes produce a normal whole of the type of fig. 30. The second sixteenth produces either a normal or a teratopthalmic whole of the general type of figs. 30 and 31. The third sixteenth may produce a normal whole (fig. 30), a teratopthalmic whole (fig. 31) or even a headless tail (fig. 32): like the third, the fourth sixteenth may produce normal or teratopthalmic wholes or headless tails, but usually the percentage of headless tails is higher than in the third.

The fifth (fig. 33), sixth, seventh (fig. 34), eighth, ninth (fig. 35), tenth and eleventh (fig. 36), sixteenths are practically always headless or else do not live long enough to undergo regulation. Moreover, they show an increasing degree of headlessness, as indicated by figs. 33–36; the length of the prepharyngeal region decreases in successive pieces and the size of the pharynx becomes less, until in the tenth and eleventh sixteenths the pharynx often fails to appear at all, *i. e.*, these pieces do not succeed in producing a prepharyngeal region or even a pharyngeal region in regulation but remain postpharyngeal.

The twelfth sixteenth may produce either a normal or a teratopthalmic whole or a headless tail. The thirteenth and fourteenth sixteenths likewise range from headless tails (fig. 37) to normal wholes, but the fourteenth produces normal wholes more frequently than the thirteenth. The fifteenth and the posterior sixteenths rarely produce anything except normal wholes.

In pieces smaller than sixteenths normal wholes are much less commonly formed, even in the anterior and posterior regions, though smaller pieces from the posterior region are capable of producing normal wholes than from any other region of the body. In general the very small pieces give rise either to tailless heads or headless tails, or they produce biaxial heads or tails, or finally they may die without regulation, a very rare occurrence in larger pieces. As regards these very small pieces, however, the difficulty of obtaining uniformity of size in isolating them is so great that the series show many irregularities. We shall return to these subminimal pieces later.

The chief results from this comparative examination of pieces are as follows:

The regional factor. The capacity for the formation of wholes differs in different regions of the body. In larger pieces the size of the head and in smaller, the frequency of head formation decrease from the anterior region to the posterior quarter of the body and then suddenly increase again. The portion of the new anterior end formed by regeneration increases from the anterior end to the posterior quarter and then suddenly decreases. The length of the posterior regenerate decreases posteriorly in wholes,

but the posterior regenerate is usually longer in headless tails than in wholes from the same region. The amount of anterior regeneration in headless pieces decreases posteriorly.

The position of the pharynx changes from near the posterior end in pieces from the anterior region to a position anterior to the middle in pieces from the third quarter of the body while in pieces from the fourth quarter it is in the middle or posterior to it as in young animals.

The process of change of shape in wholes changes in character gradually from the extreme anterior pieces to those from the third quarter, this change being associated with the decreasing size of the head.[3] In the posterior quarter, where the size of the head again increases, the process of change of shape again approaches that in the anterior pieces, but is never so extreme in character, since the disproportion in size of the head is never so extreme.

In general then we may conclude that the course and rapidity of the regulatory processes and the character of the results differ in different regions of the body.

The factor of size of piece. Regional differences in regulatory capacity appear more and more clearly as the size of the pieces decreases. The frequency of whole formation decreases and the regions of whole formation become more and more sharply limited to a part of the prepharyngeal region and the posterior quarter of the body, with decreasing size of the piece.

[3] If the contention of Morgan and others were correct, viz., that the change in shape is due, at least in part, to the migration of material to form the new parts, this change should be exactly opposite in character to what it is. In a piece which forms a large head, e. g., figs. 20, 24, 30, the region behind the head should become narrower more rapidly than other regions, since it loses so much material. Exactly the reverse should be the case when the piece forms a small head, as in figs. 22, 25 or fig. 5. But as a matter of fact, in these cases the region posterior to the head changes shape most rapidly of all. Evidently the migration hypothesis will not account for these changes.

But if we regard these changes in shape as essentially or largely due to the physical plasticity of the tissues and their mechanical adjustment to altered strains and pressures (See Child, Studies of Regulation: also Child, '09), we have little difficulty in understanding why the region posterior to the large head should be the broadest and that posterior to the smallest head, the narrowest. And it is easy to see also that the process of change of shape will differ in character and course for every combination of parts of different size.

In general the course and rapidity of the regulatory processes and the character of the result in any region of the body differ according to the size of the piece.

From these considerations it becomes evident that we cannot separate entirely the factors of size of piece and region of body in the different pieces. What occurs in a given piece from a certain region is dependent upon its size, and what occurs in a piece of given size—at least below a certain maximum—is dependent upon the region of the body from which it is taken. Moreover, as will appear later, both these factors can be modified experimentally by a variety of conditions.

4. Tabulation and graphic presentation of data

By separating a number of individuals of similar size and physiological condition into corresponding pieces and determining the number or the percentage of cases which fall under each of the groups distinguished in section 2 above, it is possible to obtain general expressions for the frequency of each form of regulation under given conditions and from these curves can be plotted if desired. In the present section certain of my results are presented in this form in order to bring out more sharply the factors which limit the regulatory capacity.

In table I the results of the most extensive group of experiments performed at one time are given. This group of experiments consisted of fifty worms all 16–18 mm. in length, all with post-pharyngeal region longer than the prepharyngeal and all collected on the same day, June 16, 1909, from the same locality and cut into pieces two days later. Of these fifty worms ten were cut into four pieces of equal length (ser. 201; fig. 17, *ae, ei, im, mq*), ten were cut into six pieces (ser. 200), ten into eight pieces (ser. 198; fig. 17, *ac, ce,* etc.), ten into twelve (ser. 199) and ten into sixteen pieces (ser. 197; fig. 17, *ab, bc, cd,* etc.), a total of 460 pieces. The old head region was of course excluded in all cases. For the purpose of direct comparison with the results of other series the results are given in the table of percentages, but since each set consisted of ten pieces, the percentages divided by ten

give the number of pieces in each case. The pieces are numbered
1, 2, 3, 4, etc., from the anterior and posteriorly. The designa-
tions of the different groups are those of section 2 above.

A glance at the table is sufficient to show the character of the
results. It is evident at once that the frequency of normal
wholes, of teratopthalmic wholes and of headless tails is a func-
tion, both of the size of the piece and of the region of the body
from which it was taken. Even with the relatively small num-
ber of pieces, *i. e.*, ten in each class, the irregularities are slight,
and my notes show that in most cases they were connected with
irregularities in the size of pieces, which cannot be wholly avoided,
even with the greatest care in isolating the pieces.

This table affords a basis for the graphic representation of the
frequency of the various groups or of any particular result of
regulation recorded. In fig. 40 the data of table 1 are presented
in graphic form so as to show the frequency of head formation
in pieces of different size and from different regions. In the figure
the ordinate represents percentages, each division being equal
to 10 per cent. The abscissa represents the size of the pieces
and the region of the body from which they are taken: it is divided
above into sixteenths and below into twelfths. Below it the
longitudinal axis of the planarian body is drawn to the same
scale with the approximate position of the pharynx and the fission
plane indicated in dotted lines. Each curve represents the fre-
quency of headformation, including both normal and teratop-
thalmic heads, at different levels of the body for pieces of a given
size. Thus the curve *ab* for the quarter pieces is plotted from
four points and is a straight line since all four pieces form heads
in 100 per cent of the cases. The curve *ac*, drawn with long
dashes shows the frequency for the sixth pieces, the curve *ad*, in
dashes of medium length that for the eighth pieces, *ae* in short
dashes that for the twelfth pieces and *af* in alternate long and
short dashes that for the sixteenth pieces.

The curves show in the clearest manner that in all except
the quarter pieces the frequency of head formation decreases
rapidly with increasing distance of the level of section from the
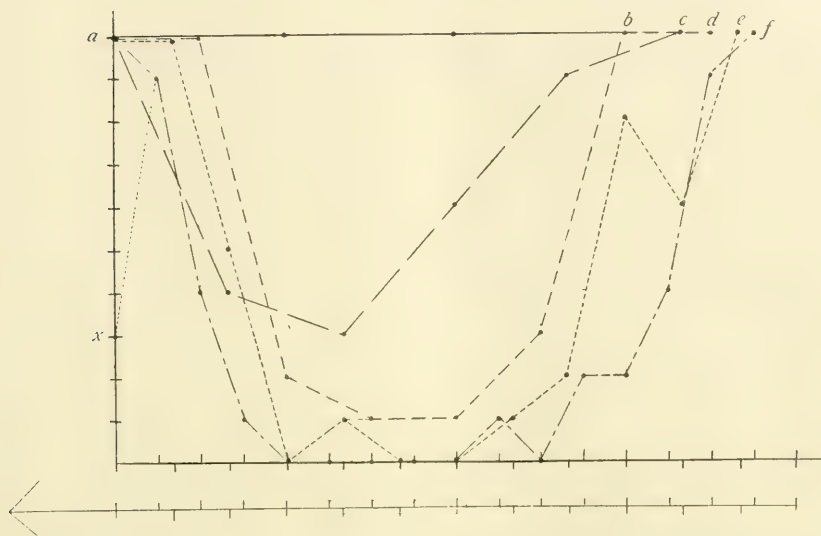
Fig. 40 Graphic representation of the percentages of head-formation in pieces of different length and from different regions of the body. The ordinate represents percentages, the abscissa the length of the pieces and the region of the body. Beneath the abscissa is drawn the longitudinal axis of a planarian body; with the positions of pharynx and fission plane approximately indicated in dotted lines and divided like the abscissa into twelfths (below) and sixteenths (above). This diagram is drawn to the same scale as the curves, so that these can be directly referred to it. The curves thus show directly the percentage of heads in pieces of different length, for each level of the body where regulation occurred. The curve *ab* (unbroken line) shows head-formation in the quarter pieces: the curve *ac* (long dashes), in the sixth pieces: the curve *ad* (medium length dashes), in the eighth pieces: the curve *ae* (short dashes), in the twelfth pieces: the curve *af* (alternate long and short dashes), in the sixteenth pieces. In all except the sixteenth pieces the course of the curves of head formation is identical with that of the curves of whole formation. The curve of whole formation in the sixteenth pieces is *xf*: its course differs from that of the curve *af* in the portion shown in dotted line. The data for these curves are obtained from table 1.

original anterior end to the middle of the postpharyngeal region and then increases rapidly.

Examination of table 1 shows that in all except the sixteenth pieces the frequency of head formation is the same as the frequency of whole formation: all the curves of fig. 40 except *af* may therefore be regarded as curves of whole formation. For the sixteenth pieces the curve of whole formation begins at *x*

TABLE 1

The character of regulation in relation to the size of the piece and the region of the body. Series 197-201. Worms 16-18 mm. in length, in good physiological condition. Begun June 18, 1909.

SERIES	PIECE	TAILLESS	NORMAL WHOLES	TERATOPTHALMIC WHOLES	HEADLESS	DEAD
Series 201. Pieces = $\frac{1}{4}$	1		90	10		
	2		60	40		
	3		90	10		
	4		100			
Series 200. Pieces = $\frac{1}{6}$	1		80	20		
	2		20	20	60	
	3		10	20	70	
	4		30	30	40	
	5		80	10	10	
	6		100			
Series 918. Pieces = $\frac{1}{8}$.	1		100			
	2		10	90		
	3			20	80	
	4			10	90	
	5			10	90	
	6		10	20	70	
	7		80	20		
	8		90	10		
Series 199. Pieces = $\frac{1}{12}$.	1		100			
	2		20	80		
	3			50	50	
	4				100	
	5			10	80	10
	6				100	
	7				90	10
	8			10	80	10
	9		10	10	70	10
	10		60	20	20	
	11		40	20	40	
	12		100			
Series 197. Pieces = $\frac{1}{16}$.	1	70	30			
	2		30	60	10	
	3		10	30	60	
	4		10		80	10
	5				100	
	6				100	
	7				100	
	8				100	
	9				80	20
	10			10	70	20
	11				70	30
	12			20	60	20
	13		20		60	20
	14		40		50	10
	15		80	10	10	
	16		90	10		

and joins the curve *af* as indicated by the dotted line drawn from *x* in fig. 40.   In other words the first sixteenth piece forms a large percentage of tailless heads instead of wholes.

It is also evident from this figure that the region in which headless tails occur is not a definite morphological region of the body, for we see that its extent increases with decreasing size of the pieces.   The various curves turned upside down would represent the frequency of headless tails.
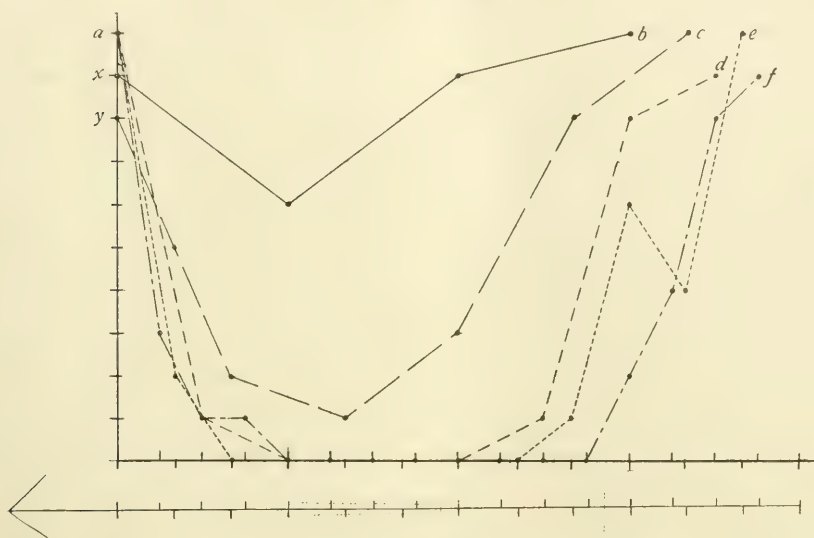
Fig. 41   Graphic representation of the percentages of "normal eyes" in pieces of different size and from different regions.   The data for the curves are from table I and the method of construction is the same as in fig. 40.   The curve *xb* shows the normal eyes at the different levels of section in the quarter pieces: the curve *yc*, in the sixth pieces: the curve *ad*, in the eighth pieces: the curve *ae*, in the twelfth pieces, and the curve *af*, in the sixteenth pieces.   Each of these curves can be compared directly with the corresponding curve of fig. 40.

In the sixteenth pieces the regions of whole formation are most narrowly limited: at the extreme anterior end of the body only 30 per cent of wholes occur, as indicated by the beginning of the curve at *x* all other pieces giving rise to tailless heads; then the frequency rises to 90 per cent in the second sixteenth but falls again to zero in the fifth and attains 100 per cent only in the sixteenth sixteenth.

Fig. 41 is constructed in the same manner as fig. 40 and shows the frequency of normal eyes for the pieces of different size from different regions. The curves for pieces of the same size are drawn in the same way in the two figures. In fig. 41 we see that the frequency of normal eyes shows in general relations of the same general character to the regions of the body and the size of the pieces as does the frequency of head formation or whole formation.

Comparison of fig. 41 with fig. 40 shows at once that the curves of normal eyes are similar in course to the curves of head formation and 'wholeness,' but it is also evident that in pieces of a given size and from a given region the frequency of normal eyes is always less than that of wholes. In other words, under similar conditions a larger piece is necessary for the formation of a whole with normal eyes than for the formation of a teratopthalmic whole. It is impossible to doubt after examination of these curves that the factors which determine the frequency of wholes as compared with headless pieces are essentially the same in general character as those which determine the frequency of normal as compared with abnormal eyes.

TABLE 2

Series 40 and 41. Worms 16—18 mm. in length, in good physiological condition. Begun July 15, 1908.
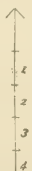
SERIES	DIAGRAM	PIECE	TAILLESS	NORMAL WHOLES	TERATOP-THALMIC WHOLES	HEADLESS	DEAD
41 (20 pieces each)		1		65	35		
		2		90	10		
		3		100			
40 (20 pieces each)		1		55	25	20	
		2		20	45	35	
		3		65	20	15	
		4		100			

TABLE 3

Sets from three series (28, 29, and 31). Worms 12—15 mm. in length in good condition.

SERIES	DIAGRAM	PIECE	TAILLESS	NORMAL WHOLES	TERATOP-THALMIC WHOLES	HEADLESS	DEAD
29 (20), 31 (20) Total 40 pieces each)	*1*	1		10	70	20	
	2	2		50	30	20	
28 (10 pieces each)	*1*	1		10	40	50	
	2	2		20	40	40	
	3	3		30	60	10	

In the following tables the data of a number of other series are given: the series numbers and the number of pieces in each set are given in the first column of each table and the diagrams in the second column show the levels of section and the relative sizes of the various pieces. As in table 1 the numbers of the different groups are percentages.

In the series 40 and 41 of table 2 the anterior part of the body is not included. The two series show the differences resulting from the separation of a given region into three and four pieces respectively. The greater frequency of teratopthalmic wholes and headless tails in series 40 is at once evident.

Table 3, from three different series, gives the results for pieces including approximately the middle half of the body: the upper half of the table shows the results for division of this region into two parts, the lower half or division into three.

The factor of size and the regional factor both appear as in tables 1 and 2. The frequency of normal wholes and of wholes in general decreases with decreasing size of the piece: even in these series, however, which do not include the extreme posterior

region, an increase in the capacity for the formation of wholes
is evident in the third set of series 28.

In table 4 a number of series including the pharyngeal and
postpharyngeal regions are combined to show the results of regu-
lation of this region as a single piece, as compared with the
results when it is separated into two and four pieces.

The results are clearly defined. The region as a whole pro-
duced 100 per cent. of normal wholes. When cut into halves
or into quarters the percentage of normal wholes decreases and
the percentages of teratopthalmic wholes and headless tails
increase. Moreover, it is of particular interest to observe that
while the whole piece produces 100 per cent. of normal wholes,
its anterior half, when isolated from the posterior half, produces
only 66.7 per cent. of normal wholes, though the same cells are

TABLE 4

From several series. Worms 12—15 mm. in length, in good condition.

SERIES	DIAGRAM	PIECE	TAILLESS	NORMAL WHOLES	TERATOP- THALMIC WHOLES	HEADLESS	DEAD
56c (10) 57c (10) 172 (10) 204 (10) (Total 40 pieces)		1		100			
178 (10) 183 (10) 186 (10) (Total 30 pieces each)		1 2		66.7 90.3	30 6.7	3.3	
182 (Ten pieces each)		1 2 3 4		30 40 70	20 30 30 30	80 40 30	

TABLE 5

From several series.   Worms 12—15 mm. in length, in good condition.

SERIES	DIAGRAM	PIECE	TAILLESS	NORMAL WHOLES	TERATOP- THALMIC WHOLES	HEADLESS	DEAD
33 (10) 88 (40) 203 (14) (Total 64 pieces)		1		50	39	9.4	1.6
32 (10) 55 (20) 56-57 (10) (Total 40 pieces each)		1 2			10 5	87.5 87.5	2.5 7.5

involved in the formation of the head in both cases.   Moreover, as series 182 shows, the anterior quarter of the piece, when isolated from the other parts, produces no normal wholes at all.

In table 5 the pharyngeal region alone is included.   Here, as in table 4 a number of series are combined.   The results of the regulation of the pharyngeal region when isolated as a whole and when separated into equal pieces are compared.

This table like the others, shows the marked decrease in the capacity for the formation of wholes with the decreasing size of the pieces.

The data presented in the above tables are all from the experiments of later years.   In addition to these my notes contain the records of several hundred individual worms which were cut into pieces from two to twenty-four in number.   It was the study of these individuals that led me to the conclusions for which the later series of experiments have merely given a more general expression.   It is impossible, however, to combine these individual records into larger series, for worms of different size were used in those experiments, the pieces were often of different length so that levels of section in different worms do not corre-

spond, and different individuals were cut into different numbers
of pieces. Since all these individual records show absolutely
nothing for our present purpose that is not more clearly shown by
the preceding tables and curves, their presentation in this paper
is quite unnecessary. Their existence is mentioned merely in
order to show that my conclusions concerning the factors of
size and piece and region of body are based on the study of a very
much larger number of individuals than is included in the records
presented above. These latter are based on 954 pieces from 314
worms, but my other data include an even larger number of
worms and pieces.

<center>DISCUSSION</center>

<center>*1. The presence of a second zoöid*</center>

Some years ago I called attention (Child, '06b) to the apparent
presence of a second zoöid in Planaria dorotocephala and P.
maculata, as indicated by the regional differences in the course
and results of regulation. The data presented above bear directly
upon this point, and it is necessary to consider the question before
proceeding farther.

The facts are briefly as follows: the capacity for head forma-
tion decreases, with increasing distance of the level of section from
the original head until at some level in the postpharyngeal region
it increases again and becomes even greater than in the anterior
region. The tables and curves show this point very clearly.
The examination of the smaller pieces, *e. g.*, the curve *af* fig. 40,
which shows the frequency of heads in the sixteenth pieces, and
similarly the series 197 of table I, shows that the region where the
power of head formation begins to increase again coincides very
closely with the region where fission occurs.

So far as I have examined planarians which do not regularly
undergo fission in this region, e. g., Planaria simplicissima
Phagocata gracilis and Planaria velata Stringer no such increase
in the ability to produce wholes, or what here amounts to the
same thing, heads, occurs in the posterior regions of those forms.

Moreover, the process of regulation in pieces from the region thus marked off as a second zoöid is similar to that in younger animals, as compared with that in other regions of the body. In young individuals of Planaria, external and nutritive conditions being as nearly as possible similar, regulation is more rapid than in older and the younger piece approaches the usual proportions more rapidly than the older; moreover, a larger piece is necessary for the formation of a whole in the older than in the younger animal. In all of these respects pieces from the posterior quarter of the body appear "younger" than pieces from other regions. As regards the rapidity of regulation no data are given except incidentally in the present paper, but the fact that pieces from the region of the second zoöid regulate more rapidly than pieces from other regions appears in every series of experiments involving these regions.

As regards the relation between regeneration in the stricter sense and redifferentiation, a comparison of figs. 38 and 39 from the region of the second zoöid, with figs. 29, 30 and 31 from the prepharyngeal region will suffice to show the differences in the relation between regenerated and redifferentiated parts in the formation of the new head, as well as the differences in the process of change of shape. Moreover, all the series which include this region and in which the pieces are sufficiently small to permit conclusions on this point, show that a smaller piece is capable of forming a whole in the region of the second zoöid than in any other region of the body.

My experiments with alcohol (Child '11b) also show that this region is physiologically younger than any other, at least at certain stages of the development of the new zoöid, and in these experiments a perfectly distinct demarcation between the intact second zoöid and the disintegrating first zoöid often appears.

And finally, my experiments on the experimental control of fission (Child, '10) add demonstrative evidence along another line to that cited above. There can then be no doubt that in individuals of P. dorotocephala and P. maculata without sexual organs and above a certain size a certain posterior portion of the body is physiologically specified as a second zoöid.

In the course of my experiments with very large individuals during the winter of 1909—10, I obtained positive evidence that in such animals four zoöids instead of two actually exist and was able in some cases to induce fission between the third and fourth zoöids before that between the second and third. In my earlier paper (Child '10) I called attention to the probable existence of four zoöids in some individuals and these later experiments afford further confirmatory evidence. As a matter of fact there is good reason for believing that the second zoöid actually consist of two zoöids in a great many cases, perhaps always, after it has attained a certain size: if, for example, this second zoöid is cut into very small pieces we commonly find that the regional distribution of the regulatory capacities resembles that in the whole individual from which this zoöid was taken. A decrease in the power of whole formation occurs with increasing distance of the level of section from the zone of fission to the posterior quarter of fifth or the zoöid where it increases again. In short, if we cut the second zoöid into sufficiently small pieces it appears as a miniature of the whole animal.

The fact that the zoöids in Planaria do not become visibly morphologically differentiated before fission, is at least in part the result of the physical consistency of the planarian tissues which is such that independence of motor reaction brings about separation before the degree of physiological isolation is sufficient to permit the differentiation to become visible. The result of inhibition of fission will be discussed at another time.

If Driesch's view that structure is developed for function, not by it (Driesch, '05, etc.), a view which he has expressed very positively in opposition to various observations and conclusions of mine, is correct, the existence of such a region in the planarian body, functioning like a second younger animal, but without any of the visible morphological characteristics of such an animal would seem to be impossible. Yet there can be no doubt that the second zoöid is there and that in certain respects and to a certain degree it functions as an independent animal. I believe that here as elsewhere in organisms, visible structure is merely the record of past functional $i.\ e.$, dynamic activity of some kind

or other.   This second zoöid in Planaria is an excellent illus-
tration of the fact that a new organism developing from a part
of an old one may attain a certain clearly distinguishable degree
of physiological individuality before it becomes morphologically
recognizable.

2.   *The question of 'wholeness'*

The whole in regulation is often sharply contrasted with a partial
result as if the two were separated by some hard and fast natural
distinction.   It is of course true that in many cases there is not
the slightest difficulty in distinguishing between a whole and some-
thing that is not a whole.   On the other hand there is not the
least reason to believe that all 'wholes' are alike or even that
they possess the same degree of 'wholeness': in fact the data of
variation show us very clearly that they differ from each other in
a multitude of ways and that there is no clear distinction between
wholes and not wholes.   The notion applied to organisms of
the whole as opposed to the not whole is like other human con-
ceptions in that it corresponds to no clearly defined and limited
group of objects in nature, but is merely a grouping made from
individual or collective experience for convenience of thought.
   In the above described experiments on Planaria we can
readily distinguish certain results of regulation as not wholes,
but between these and what for convenience we term normal
wholes there are all possible intermediate gradations.   In the
process of head formation, for example, we see all stages of
completeness from what we have termed headless, on the one
hand, to normal heads on the other.   But there cannot be the
least doubt that these normal heads also differ from each other
in greater or less degree and in various ways.
   Moreover, in examining the course of regulation we find that
different pieces attain wholeness in very different ways.   The
pieces from different regions of the body illustrate this point.
Driesch has designated such regulations as "equifinal" but this
term is like the word "wholeness" merely a convenient notion.
There is not the least reason for believing that pieces of Plan-

aria in which the course of regulation has been different attain
identical results. To call such regulatory processes equifinal is
simply to beg the whole question. Certainly the general morpho-
logical similarity affords no adequate grounds for such conclu-
sions. We have much more reason to believe that the results
are not alike in any two pieces of Planaria, and I hope to show in
later papers that they actually are different in at least certain
cases where "wholes" are formed.

The visible morphological structure of the organism is, so to
speak, merely a very incomplete record of the dynamic processes.
Only the most characteristic, the least variable, the most endur-
ing or the most intense leave records sufficiently well marked to
appear as visible structure. Two organisms may be indistin-
guishable or almost indistinguishable as regards their general
form and structure and still be widely different from each other
in many respects.

And again, the distinction between wholeness and incomplete-
ness is likely to lead us astray in another direction. It becomes
easy to regard the formation of a whole from a piece as 'success'
and the formation of a partial structure as 'failure'. But when
we do not impose such notions upon nature as rigid categories we
must recognize the fact that success and failure are not involved.
Every living system is as much a success as any other. It is
simply the product of a certain constellation of conditions, internal
and external.

In the investigation of the regulations of form and structure in
organisms the stress has too often been laid upon wholeness to
the more or less complete exclusion of other results. It is often
said that any piece, or almost any piece from the body of Plan-
aria produces a new whole. How far this is from being true the
above described experiments show. Strictly speaking, the limits
of wholeness in the regulation of this form are very narrowly re-
stricted. So far as our experiments go at present, the formation
of a 'whole' in Planaria is possible only in pieces above a certain
size which varies with the region of the body and with various
external conditions. Manifestly this process of formation of a
whole is immediately dependent upon the kind and the rate of

metabolism, upon the character and degree of physiological corre-
lation between parts and it is also limited, as will appear later by
external conditions, though these limits are not constant, but
vary with the internal factors. For example, larger pieces from
a given region will produce wholes at a lower temperature than
smaller pieces. Certainly the facts in regard to Planaria as we
know them at present afford no basis for the assumption of an
entelechy.

3. The 'potences' of parts

It is sufficiently evident from the above experiments that the
regulatory processes in the cells at a given level of the body
differ in degree and kind, according to their relations with other
cells. If for example, we isolate the second fourth of the body
(*ei*, fig. 17), we find that in well nourished worms it produces a
head at its anterior end and this head is commonly normal
(table 1, series 201, no. 2). If, however, we cut it transversely
in half, both of its halves produce heads only rarely and these
heads show abnormal eyes (table 1, series 198, nos. 3 and 4).
Again, if we cut the original quarter into fourths, none of these
produces a new head (table 1, series 197, nos. 5–8). Evidently
the reaction of the cells at the anterior end of the original piece
is dependent, at least as regards its intensity, not merely upon
these cells alone, but upon the cells of all levels of the piece, for we
find that with the successive removal of cells from the posterior
end of the piece, the ability of the cells at the enterior end to form a
head decreases and disappears. In a later paper I shall show that
the rate or intensity of the dynamic processes is an important
factor in head formations.

What shall we say then concerning the potences of these cells
at the anterior end of the piece? We must of course admit that
they are potentially capable of forming a head, but the experi-
ments demonstrate that such an assertion means nothing very
different from the statement that an acid is potentially capable
of forming a salt. In both cases we mean merely that a certain
reaction is possible under certain conditions. But the mechan-

ism for this reaction does not exist wholly in the acid, nor does
itexist wholly in the cells at the anterior end of the above piece
it exists in the acid plus the metal and in these cells plus other
cells.   Driesch's attempt to reduce the 'machine theory' to
absurdity by the argument that since any part of a harmonious
equipotential system is capable of producing any other part,
therefore, according to the machine theory, each part must repre-
sent at all times all possible parts of an infinite number of machines,
which is manifestly absurd, is itself absurd because no such
assumption is necessary.   A given part represents one machine,
or a component of such a machine, at a given time; at another
time if its relations to other parts have changed, it may repre-
sent another machine or component.   To maintain that such a
part possesses the same potences under these different conditions
means simply that its constitution is capable under the proper
conditions of changes which make possible a certain different
series of reactions: it certainly does not mean that it possesses
the same constitution at all times.   At any given time the consti-
tution of parts which are equipotential in this sense may differ
widely.

But I believe it is misleading to designate such parts as equipo-
tential.   As parts of a system at any given time they are not
equipotential, for they possess different constitutions as the facts
show, in short the piece of Planaria which is capable of regula-
tion is never an equipotential system.   The more closely it
approaches to equipotentiality, the less capable of regulation, or
indeed of life it becomes.   The apparent equipotentiality in
some eggs and in some of the lower organisms is undoubtedly
merely apparent, in so far as these forms are capable of develop-
ment or regulation.   As in the case of Planaria, it will disappear
as soon as isolation of parts and analysis of results is extended
sufficiently far.   I think we may say that there is at present no
valid evidence for the belief that any living system which is
undergoing regulation or development in nature is at any given
time an equipotential system.   We cannot properly speak of
the potentialities of parts unless we assume that their constitu-
tion remains constant: a change in constitution means a new part

and new potentialities. And if we distinguish between 'explicit potences' those resulting from a given constitution, and 'implicit potences,' those resulting from possible changes in constitution, we gain nothing for if our conclusions concerning the physical world are of any value, there is good reason to believe that the implicit potences of all systems in nature are the same within certain limits. Science can deal directly only with explicit potences, and it is only by making implicit potences explicit that we can actually know anything about them. After such knowledge is once gained, however, it may serve as the basis for prediction.

To return to Planaria, the facts show that the morphogenic reactions of a given part, depend within certain very wide limits upon its physiological correlation with other parts. Planaria is not then an equipotential system in Driesch's sense. I believe that investigation, unless limited by technical and other difficulties which make exact experimentation impossible, will demonstrate the same to be true for all other so-called equipotential systems. I do not mean to exclude the possibility that such systems may approach or become at times equipotential aggregations—strictly speaking they cannot be called systems when in this condition—but I believe that the occurrence of the process of development in nature or of regulation in experiment is in itself proof that the system concerned is not at the time equipotential. I have shown above for Planaria that with sufficiently extended and exact investigation of the phenomena of form regulation the apparent equipotentiality disappears. And if the piece undergoing regulation is not equipotential in its various parts, what need is there for an entelechy?

4. Preliminary analysis of the factor of size of the isolated piece

It is evident from the above experiments that both the course and the results of regulation are related in various ways to the size of the piece. In the first place, a gradual change in the character of the result occurs with change in the size of the piece, e. g., in certain regions of the body the head becomes less and less

"complete" with decreasing size, and finally the piece becomes
headless: similar changes also occur with respect to the posterior
end in other regions. In general we may say that with decreas-
ing size the piece gradually loses its capacity to form a whole and
forms a part instead.

Secondly, changes in the method of regulation are often asso-
ciated with change in size of the piece. Of the two pieces with
anterior ends at the same level, the shorter piece shows a rela-
tively larger amount of regeneration in the formation of the new
anterior end. A comparison of fig. 19, a posterior half (*iq*, fig.
17), with fig. 22, the third quarter (*im*, fig. 17), illustrates this
point. In fig. 19 the new tissue ends just posterior to the eyes,
while in fig. 22 a considerable region posterior to the eyes is
formed from regenerated tissue. The general rule is that the
shorter the piece from a given region of the body, the greater the
relative amount of regeneration as compared with redifferentiation
i. e., the less the ability to reproduce the missing parts without
extensive outgrowth. This rule holds good only so far as simi-
lar structures are concerned. *i. e.*, for heads or posterior ends
but not for headless or tailless pieces as compared with wholes

Thirdly, within certain limits, decrease in the size of a piece
from a given region of the body is accompanied by a decrease in the
rapidity of regulation. This rule holds only for pieces below a cer-
tain size which is different for different regions. If we begin with
whole animals and remove from one a small portion, from another
a somewhat larger portion and so on, we shall find that at first
the rapidity of regulation increases as the size of the regulating
piece decreases, *i.e.*, as more and more is removed from it. From
a certain size downward, however, the relation is reversed and
the smaller piece regulates less rapidly than the larger. The
size where this reversal of relations occurs, *i.e.*, what we may call
the critical size corresponds rather closely with the size where
teratopthalmic wholes begin to appear.

And finally, the death rate of the pieces varies inversely as the
size of the piece. Among the larger pieces there are usually no
deaths, except under extreme external conditions, but below a
certain size the death rate increases rapidly and this size differs
for different regions of the body. In table 1, for example, no

deaths occur among the one-fourth, one-sixth and one-eighth pieces: in the twelfth pieces the death rate is low and is confined to the pieces from the middle region of the body. In the sixteenth pieces the death rate is distinctly higher, though even here deaths do not occur among the pieces from the terminal regions. Tables 2, 3 and 4 show no deaths at all, but in table 5, where the pieces are smaller than in tables 2—4, deaths again appear and are more frequent in the smaller than in the larger pieces. Evidently the possibility of maintaining an independent existence is a function of the size of the piece.

The presentation of further data must precede any extended consideration of this factor of size. At present it is desired to call attention only to certain points.

In the first place, the change from wholeness to partial structures with decreasing size suggests that below a certain limit of size something necessary for wholeness is lost. What this something is will appear more clearly later.

The change in the method of regulation with decrease in size indicates a change in the physiological condition of the cells near the cut surface. In general we find that under like conditions redifferentiation occurs in parts which are more closely similar to the part removed, regeneration in parts less similar (Child, '06a)[4]. In other words, in the former case redifferentiation takes place more rapidly and the stimulus to regeneration is less than in the latter. If my conclusions are correct on this point then it is evident that with decreasing length of the piece the

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[4]Various conditions alter the relation between redifferentiation and regeneration. For instance, as Morgan observed, starvation increases the relative amount of redifferentiation. Various depressing agents, such as anesthetics, also increase the relative amount of redifferentiation, until in extreme cases regeneration, in the formation of a head for example, is almost absent. In all cases there is evidently an inverse physiological correlation between the two forms of reaction. The formation of new tissue is retarded in the absence of nutrition or in the presence of agents which retard the oxidations, consequently the process of redifferentiation proceeds relatively more rapidly than otherwise. In general the result in any given case is determined by the relative rapidity of the two processes. All conditions which retard the rapid proliferation of cells and the growth of new tissue, as well as all conditions which accelerate redifferentiation (e. g., physiological likeness) will increase the relative amount of redifferentiation in a given case, and vice versa

cells of a given level become less and less capable of the regula-
tory reaction which leads to formation of a whole. I believe that
this is strictly true, moreover, this conclusion is in complete
agreement with what occurs as regards the character of regula-
tion with decreasing size of the piece. We see pieces becoming
less and less capable of forming wholes and it is evident that this
decreasing capacity is not due to lack of material, for in many
cases the partial structure which such pieces form apparently
requires as much or more material than the formation of
whole and, what is still more striking, we often find that a small
piece which produces a partial structure in the first regulation,
will, after another operation at the incomplete end, form a normal
whole. In a headless tail, for example, the anterior end is physio-
logically more capable of head formation after the first regula-
tion of the piece is completed, even though it does not form a
head, and if we remove the anterior end a head will be formed in
the same manner as in pieces from the anterior region of the body.
These pieces will be considered more fully in another paper.

Furthermore, the decrease in the rapidity of regulation with
decrease in size below a certain limit is not due to increasing
difficulty in obtaining the necessary material, for such pieces
often accomplish much more extensive growth than larger pieces.
In a later paper I shall show that a decrease in size of the piece
is accompanied by a decrease in rate or intensity of the dynamic
processes and that the decrease in regulatory capacity is directly
connected with this change.

Lastly the increasing frequency of death with decreasing size
of the piece points to the conclusion that not only the ability of
the pieces to form wholes, but their ability to remain alive is
dependent to a considerable degree upon the correlation between
parts of more or less different character.

All the facts then point toward one general conclusion which
may be stated briefly here, viz: that *the capacity of the cells or
parts at any level of the body is dependent, not merely upon their own
constitution, and upon conditions which permit continued existence
but upon physiologic correlation with other parts more or less dif-
ferent from them.* As the length of a piece of Planaria decreases,

the piece becomes less and less 'totipotent,' until a limit is reached, below which it is capable of producing only a part. In short, the changes in the regulatory capacity with decrease in size of the piece are essentially the result of decrease in physiologic correlation between parts: in other words, the shorter the piece the less its dynamic activity. We shall return to this point in a later paper in connection with other data.

5. *Preliminary analysis of the regional factor in regulation*

The question as to the existence of the second zooid has already been considered. For the present we may limit our consideration to the first zooid, *i.e.*, the region from the anterior end to about the middle of the postpharyngeal region. The regional differences in this first zooid are briefly as follows: in pieces of the same length below a certain limit the capacity to form a head decreases from the anterior to the posterior end of the body, and conversely the capacity for tail-formation decreases from the posterior end anteriorly. In pieces sufficiently small the power to form a head disappears completely in the posterior one half or one third of the body and the power to form a tail in the most anterior regions behind the head; in the one case headless tails, in the other tailless heads arise from isolated pieces. With still further decrease in the size of the pieces the regions of head and tail formation become still more narrowly limited.

The position of the pharynx in pieces from different levels shows that the primordium of the new prepharyngeal region is longest in the anterior regions of the zoöid and decreases in successive pieces to the posterior end. Furthermore, the relative amount of anterior regeneration is least in the most anterior piece and greatest in the most posterior and the relative amount of posterior regeneration is greatest in anterior and least in posterior pieces. The rapidity of head formation is also greatest in the most anterior, least in the most posterior pieces and the rapidity of tail formation is least in the most anterior pieces, but in the posterior pieces it is more or less closely correlated with the rapidity of head formation. And finally, in pieces below

a certain size the death rate increases toward the posterior end
of the first zoöid, *i.e.*, independent existence is possible in smaller
pieces from the anterior than from the posterior regions of the
first zoöid.

All of these facts indicate that a graded difference of some sort
in the dynamic processes exists along the axis, and together with
the results concerning size of pieces, the experiments show, first,
that a certain minimal portion of this graded difference is neces-
sary for the regulation of a piece into a whole, and second, that
the rate of gradation along the axis differs in different regions of
the body. In other words, the minimal length of pieces which
are capable of forming a whole is different in different regions
of the body. In a later paper, however, I shall show that these
factors are by no means constant, but depend, at least in large
degree, upon the rate of metabolism or of certain metabolic pro-
cesses.

It is this dynamic gradation along the axis, together with the
complex of correlative conditions associated with it, which I
regard as constituting physiological polarity. According to this
idea polarity is not a condition of molecular orientation, but is
essentially a dynamic gradient in one direction or different grad-
ients in opposite directions along an axis, together with the condi-
tions, and particularly the physiologic correlation between
different parts along the axis, which must result from the existence
of such a gradient or gradients.

The change from the production of wholes to partial structures
with decreasing length of piece constitutes strong evidence in
favor of this view, while on the basis of a molecular hypothesis
of polarity it is difficult to understand why short pieces should
not produce wholes as well as long pieces, if they produce any-
thing.

The results of these experiments also indicate clearly that the
dynamic gradient is not uniform but differs at least quanti-
tatively in different regions of the body. A molecular hypothesis
of polarity affords us no basis for understanding why the minimal
piece for the formation of a whole must be larger in some regions
than in others.

And finally, I wish to emphasize the point that polarity may conceivably consist either in a single gradient in one direction or in two gradients in opposite directions. At present we know nothing concerning the nature of the differences between the metabolic processes concerned in the formation of head and tail in Planaria, but certainly the two parts are, to a considerable extent the result of differences in arrangement and distribution of similar components. We find one of these capacities decreasing in one direction, the other in the opposite direction: morphologically the results at the two poles are qualitatively different organs, but what the differences in the dynamic processes which form these organs may be does not appear from the experiments described.

According to the above view then, a certain minimal fraction of the axial gradient or gradients is necessary in every case for the formation of a whole. But it is necessary to call attention not merely to the existence of the axial gradient, but to the correlative factor in polarity.   Morgan's hypothesis of the gradation of substances possesses certain features in common with my idea of a metabolic gradient, but the assumed gradation of substances is more stable and requires the assumption of migration; the most unsatisfactory feature of this hypothesis, however, is its failure to recognize the correlative factor in polarity. The gradation of substances alone cannot account for the fact that the same cells give rise under certain conditions to a head, under certain others to a tail, but when we consider that a gradation of any kind along the axis may give rise to a variety of correlative conditions, the phenomena become less puzzling, and we see that the directive or apparently directive feature of organic polarity is in reality a matter of physiologic correlation.   In many cases and in various degrees we see the processes at the different levels of the body, or at the two ends of a piece exerting an effect upon each other, or one upon the other.   For example, in pieces from the extreme anterior region just behind the head of the first zoöid, the formation of a head occurs, even though no pharyngeal or post-pharyngeal region is formed, i.e., the constitution of these pieces is such that they can produce a head without correlative influence

from posterior portions of the body. In the posterior region of
the first zoöid, however, the correlation with posterior regions is
necessary, under the usual conditions, for the formation of a head.
But when a head has once formed at any level of the original
body we find that the capacity of the adjoining regions for head
formation has been correlatively increased. In general we may
say that in regions adjoining a previously existing head the process
of head formation after isolation approaches more or less closely
to 'self-differentiation,' or as I prefer to call it, 'constitutional
differentiation,' while the correlative factor in head formation
increases in importance with increasing distance of the level from
the previously existing head. Similar relations obtain in the
formation of posterior ends: in the posterior region of the first
zoöid the formation of the tail is much more nearly a constitu-
tional process, while in pieces from the anterior end of this zoöid
it is to a much greater extent a process of correlative differenti-
ation. These differences mean, I believe, that the regions adjoining
a head possess a certain constitution in consequence of their posi-
tion in the body, *i. e.*, in consequence of the correlative effects to
which they are subjected. This constitution is such that all that is
necessary for the formation of a head by these parts is the isolation
of a sufficiently large mass from the previously existing head,
irrespective of whether posterior regions are present or not.
If these conclusions are correct then it becomes less difficult to
understand why the formation of a new head at a level far pos-
terior to the original head can occur only when a considerable
region posterior to it is present. Before section this level was
in no way closely associated with the old head and the effect of
correlative factors upon it in the original body was quite different
from that upon the anterior regions. But when we cut the
body in such a manner that this level lies at or near the ante-
rior end, then the cells adjoining the wound react to the altered
conditions by loss of specification and growth, but the final re-
sult is dependent in part upon the more posterior levels with
which these cells are correlated. The question as to whether
this correlation determines the character or quality of the reac-
tion or merely its rate or intensity will be discussed in connec-

tion with other experimental data.   As regards the development
of a posterior end the case is somewhat different for in this species
correlative factors always play an important part in its forma-
tion.

The experiments show beyond a doubt that there are two
factors which vary or exhibit a gradation along the axis, viz.,
constitution, or what is essentially the same thing, metabolism
and its structural colloid field, and physiologic correlation.   And
there is no evidence to indicate that the phenomena of polarity
depend on anything but these two factors.

6.   Heredity in the regulation of pieces

Extended discussion of this aspect of my experiments must be
postponed until further data are presented.   Here attention may
be called to a few points, some of which are already familiar,
while others are less generally recognized.

That the pieces inherit their capacity to form wholes, or, for
that matter, to form anything, is, I think, sufficiently evident.
The experiments show, however, that there are limiting factors
in such inheritance which are associated with the size of the
reproductive element and with its position in the body.   In the
preceding sections the attempt has been made to analyze these
factors of size and region into terms of organization, with the
conclusion that any piece must possess a certain minimal organi-
zation along the axis in order to be capable of producing a whole.
In the cases which we have considered this organization is directly
inherited as a fraction of the organization of the whole animal.
In a later paper we shall consider the question as to whether it
may under certain conditions arise in other ways.   This axial
organization is not directly related to the visible morphological
structure and is not necessarily anything very complex: as sug-
gested above it is probably a dynamic gradient or gradients with
the accompanying correlative factors.   In short it may be of
essentially the same character as the polarity of the egg.

The conditions which determine the formation of normal and
teratopthalmic wholes, as described above are of considerable

interest in this connection: the experiments show that even in the case of organs of such definite form and localization in the body as are the eyes, the position, form and number of the organs may be dependent upon dynamic conditions in the system as a whole, rather than upon any specific or localized hereditary element.

In general I think we may say that so far as these experiments go they indicate that what is inherited is merely capacity for reaction, and that such capacity consists in the constitution and the correlation of parts of the reproductive system or element. The same 'character' may in one case be chiefly the result of constitution of a localized part, in another it may be largely the result of correlation between different parts. But in the cases of constitutional inheritance there is good reason to believe that correlation with other parts in the past has played an important part in bringing about the particular constitution concerned. That is to say, this constitutional factor in inheritance cannot be regarded as in any sense the fundamental factor, in other words, we cannot regard the reproductive element as originally a mosaic of localized hereditary qualities. In all cases where it approaches this form, there is reaason to believe that the existing specification is the result of a specification or differentiation which has already taken place and in which both constitution of parts and correlation between them have played a rôle. In more general terms, the reproductive element may be in very different stages of development at the time when it is isolated from the parent organism.

The experiments show that any piece of Planaria capable of development into a whole, or into anything with definitely localized parts is not an equipotential system. I do not believe that any reproductive element which is capable of development is such a system: organization, i.e., different constitution of different parts and physiologic correlation between them to a greater or less extent, is necessary for development.

These conclusions and suggestions apply primarily to inheritance in the pieces of the planarian body, but it seems probable that there is no very fundamental difference between such a

piece and the sexual reproductive element except that the latter is so highly specified physiologically that it requires special stimulation to initiate the new series of processes while in the pieces of the planarian isolation alone is sufficient. While I should not for a moment maintain that the investigation of inheritance in experimental reproduction can take the place, or obviate the necessity of other methods of attack upon the problem of heredity, yet I do believe that in such forms of reproduction we have the problem given in somewhat simpler terms than in sexual reproduction and furthermore the possibility of controlling the size of the reproductive element, the region from which it shall arise and the conditions of its development, as well as the metabolic conditions in the parent, afford various possibilities of experimental analysis which are not given in sexual reproduction in the higher animals. Believing that wherever reproduction occurs in the organic world, there inheritance occurs also, I have for years past regarded the field of 'form regulation' as of great importance for the problem of heredity, though this feature has not been emphasized in my published work thus far. But having now acquired as I believe, a fairly satisfactory basis for future investigation, I have felt it desirable to present my experimental data with some regard to their bearing upon the problem of heredity. The present paper is intended primarily to serve as a foundation for what is to follow.

SUMMARY

1.   In Planaria dorotocephala (also in P. maculata) two zoöids are commonly present during the asexual period, a longer anterior and a shorter posterior. The presence of these zoöids is indicated by certain regional differences in the character, method and rapidity of regulation of isolated pieces.

2.   In the anterior zoöid the character, the method and the rapidity of regulation are dependent upon the length of the piece and upon the region of the body from which it is taken.

3.   The constitutional capacity for head formation decreases from the anterior regions of the first zoöid posteriorly the capacity for tail formation from posterior regions anteriorly, but correlative factors may determine that heads or tails arise at levels of the body where the constitutional capacity is slight.

4.   The capacity to produce a 'whole' decreases with decreasing length of the piece, until in pieces below a certain length, which differs in different regions of the body, partial structures instead of wholes arise. There is no sharp limit between 'wholes' and partial structures (e.g., tailless heads or headless tails.)

5.   The size limit in the formation of wholes and the appearance of partial structures in pieces below this size limit indicate that polarity consists essentially in a dynamic gradient or gradients along an axis, together with the correlative factors resulting from such a gradient. According to this hypothesis, wholes arise only from pieces which include a certain fraction of the gradient or gradients.

6.   That the dynamic gradient is not uniform is indicated by the fact that longer pieces are necessary for the formation of wholes and even for continued existence in some regions of the body than in others.

7.   'Potence' is not necessarily inherent in the part involved. The head-forming potence of certain pieces, for example is shown to depend, not merely upon the parts directly involved in the development of a head, but upon the piece as a whole. We can even determine whether a head shall or shall not form at the

anterior end of a piece by including in the piece or removing from it a certain amount of tissue at its posterior end.

8. The development of a new organism or a new part from an isolated piece of the planarian body is as truly a matter of heredity as is the development of an organism from a fertilized egg. Moreover, the possibility of controlling experimentally the occurrence of the reproduction, the size of the reproductive element, the region of the body from which it is taken, the physiological condition of the parent and the conditions under which the development of the isolated reproductive element shall occur permit us to attack the problem of heredity in some ways that are impossible in connection with sexual reproduction in higher forms. It must also be admitted that in these regulating pieces the problem of heredity is given in relatively simple terms and we may hope by cautious analytic investigation of such cases to throw some light on the more complex phenomena. Some conclusions and suggestions from the experiments which bear upon the problem of heredity are briefly stated in the text, but extended discussion of this phase of the subject is postponed until after further data have been presented.

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CONTRIBUTIONS TO THE PHYSIOLOGY OF REGENERATION

IV. REGULATION OF THE WATER CONTENT IN REGENERATION

SERGIUS MORGULIS

SEVEN FIGURES

CONTENTS

THE CURVE OF REGENERATION AND THE CURVE OF GROWTH

Recent studies on regeneration have revealed the fact that the process is divisible into a series of stages, marked by different degrees of intensity of the regenerative energy. This periodic change in the regenerative energy in the course of the regeneration of an organ was discovered independently by Miss Durbin ('09) and myself ('09) while experimenting with different organisms and with different objects in view. The close similarity of the results of both studies is especially noteworthy, since the quantitative determinations were based upon the amount of differentiation of the regenerating organ in the former case, and upon the amount of its elongation in the latter. But regardless of the difference of

method of studying and of the difference of the animals experimented upon (annelid and amphibian), the general conclusions drawn from both investigations are practically the same, except perhaps on some special points.

My investigation of the energy of regeneration of the tail in the polychaet Podarke obscura, based upon the degree of differentiation as determined by the number of regenerated segments, led to establishing four distinct, though not sharply defined, stages in the process of posterior regeneration. The first stage, during which the wound closes over but the proliferation of the new organ has not yet begun, is of very short duration, the length of time depending upon the individual and also upon the level of the cut. The second stage is marked by a very rapid formation of new segments; the third stage, during which new segments are still formed at a high rate, is nevertheless characterized by a sudden fall in the regenerative energy. These stages, two and three, extend over a period of about two weeks; the ultimate size of the regenerating tail attained at the completion of the process of regeneration practically depends entirely upon the progress made during this period. The fourth and last stage is one of slow but continuous decline of the regenerative energy until the zero point has been reached. This succession of stages in the regeneration of the tail is represented by a curve, fig. 1, constructed according to the data contained in tables 2 and 3 in the first paper of these contributions to the physiology of regeneration (Morgulis '09).

Miss Durbin ('09), experimenting on tadpoles of Rana clamitans (Latreille), found that the new growth of the tail presents four definite stages, essentially like those outlined above. The correspondence of her results with those obtained on Podarke, apart from the weight it lends to the conclusions, is also important as indicating that either the degree of differentiation or the amount of growth of the new organ may be equally relied upon in studying the nature of the regenerative process.

Below I reproduce one of Miss Durbin's curves (fig. 2) showing the successive modulations of the intensity of tail regeneration in tadpoles, in order to emphasise its close similarity to the curve of tail regeneration in the worm (fig. 1). This curve was plotted on

the basis of the average data for a number of tadpoles with an average body length of 49.1 mm. The average amount of tail removed was 14.8 mm. and the average amount of regenerated tail was determined for a period of 38 days at intervals of about four days each.

Comparing the two curves we observe, first of all, that even in organisms so widely apart in the scale of classification as are the
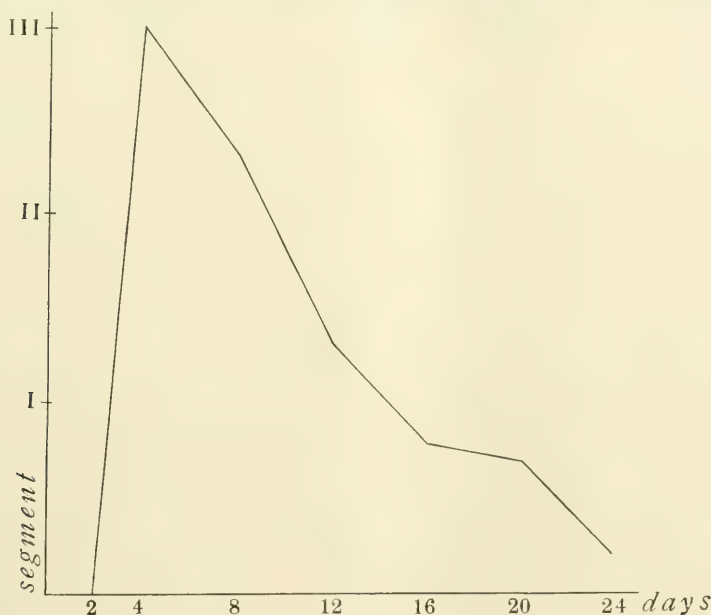
Fig. 1 Curve showing the rate of regeneration of the tail of Podarke obscura, as expressed in the number of segments regenerated during the first twenty-four days following operation, based on the data of tables 2 and 3 in Morgulis, '09.

annelids and the amphibians, the rate of posterior regeneration obeys apparently the same natural law, which therefore transcends specific and generic and even greater differences. In both instances we find that the bulk of regeneration is accomplished within a fortnight, between the second and fourteenth day succeeding the operation,—during which time regeneration reaches its climax and suddenly declines; subsequently the rate of regeneration continues to decrease slowly but steadily.

The curves of posterior regeneration obtained for Podarke and
for the tadpoles of Rana clamitans present a striking parallelism
to the curves of growth of various animals worked out by Minot
('07), and to this paralellism I wish now to direct attention.
Minot's curve is constructed upon the principle that the growth
for each successive period may be expressed as a fraction of the
amount of growth for all preceding periods; *i.e.*, the size at the
beginning of the period under consideration; it always shows a
rapid rise at first, then falls abruptly, and later continues to slope
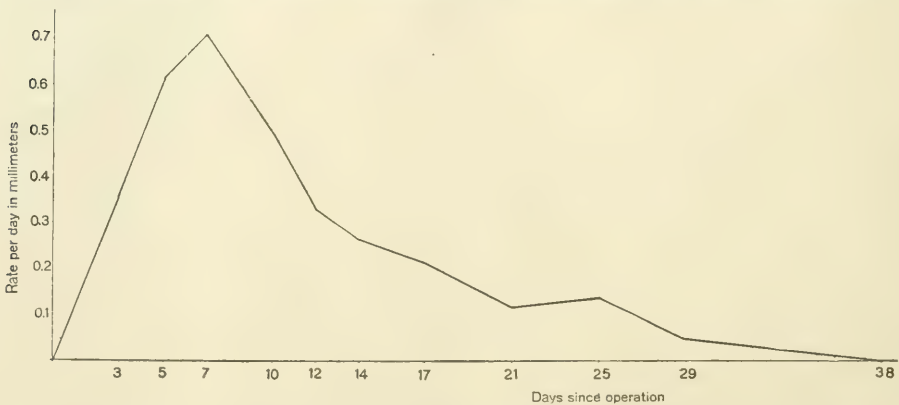
Fig. 2   Curve showing the rate of regeneration of the tail of tadpoles of Rana
clamitans Lat., as expressed in the daily increase in length during thirty-eight
days following operation.   Copied from Durbin ('09, p. 409, fig. 3) The Journal of
Experimental Zoölogy.

down gradually, reaching practically the zero point. If we
examine any of the curves of growth given by Minot, for instance,
fig. 21 on page 197, reproduced below, it will be seen that the
power of growth, very slight at first, gathers great strength two
or three days after birth. Rapid growth lasts only a few days, then
the rate decreases, at first, with notable rapidity, but after
forty-five days only very slowly.

It is unnecessary to dwell at length upon this parallelism be-
tween the curves of the rate of regeneration and those of the rate
of normal (formative) growth, which is made evident by a com-
parison of figs. 1, 2, and 3.   The curves are all of fundamen-
tally the same form.   The rapid fall in the curves succeeding the

maximum rate may be due either to the greater impediment resulting from the increase in the size of the growing mass, or to the fading out of the original impetus, which had caused the immense output of growth energy near the start, or perhaps to both factors combined. The principal interest in this connection is that in both normal and regenerative growth the maximum intensity of the process is not attained at the very beginning and is retained for only a comparatively short time.
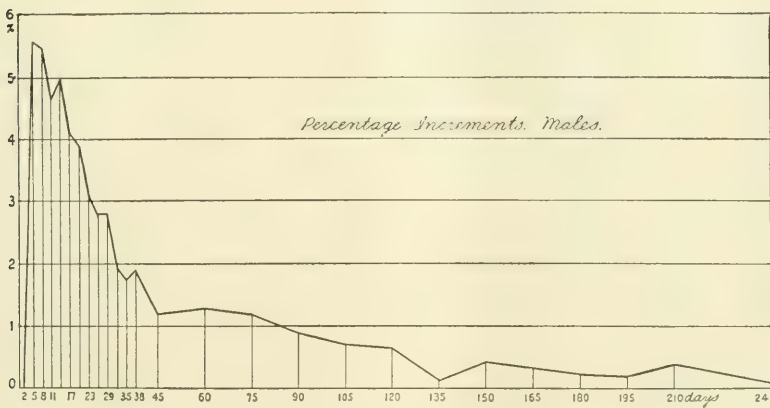
Fig. 3 Curve showing the daily percentage increments in weight of male guinea pigs. Copied from Minot ('07, p. 197, fig. 21). Popular Science Monthly, September, 1907.

THE RÔLE OF WATER IN GROWTH

Long before zoölogists had come to realize that there was a blank in their knowledge of the physiology of the organism so far as phenomena of growth are concerned, botanists had already thoroughly investigated the problem of plant-growth. It was J. Loeb who, following the example of botanists of Sachs's school, first attempted to analyze the relation of osmotic pressure to animal growth, and thus brought this question to the front. Although later investigations of the influence of osmotic pressure upon regeneration are somewhat at variance with Loeb's conclusions relative to hydroids, yet it should be remembered that his

work forms the starting point of a series of researches which have thrown light upon the problem of animal growth.

The rôle of water in the processes of growth was first definitely appreciated by botanists. An analysis of the water content in Heterocentron at different levels of the stem, for instance, revealed that, while at the tips there is 73 per cent of water, the amount of water increases suddenly in the first internode to 88 per cent, and in the second internode reaches a maximum of 93 per cent, and that in the following internodes the per cent of water commences to fall off. Davenport ('97), inspired by the results of botanists, extended the investigation of the change of the water content during growth to animals. He used for that purpose frog embryos, studying the water content at different stages by the ordinary method of dessication, and found that twenty-four hours after hatching the per cent of water was 56, that the percentage of water increased steadily up to the fourteenth day after hatching, when the maximum (96 per cent) was reached, and that it then began to diminish, being only 88 per cent eighty-four days after hatching. These results are graphically represented in fig. 4, which is reproduced from Davenport's original paper. It shows that very rapid increase of the quantity of water, followed by a slow but continuous fall in the water content as one passes from the earlier to the more advanced stages of development.

While this investigation of Davenport, emphasizing the rôle of water, has helped greatly to further the study of growth, and in fact has evoked several very important contributions from other students, it may nevertheless be pointed out that the discovery in young embryos of a greater water content than in old ones had been already demonstrated by Bezold ('57) for a number of animals, including the amphibians. Bezold (p. 523) says "Die Entwicklung und das Wachsthum eines jeden Thieres ist durch gewisse, für die Art oder Gattung desselben typische Veränderungen in dieser Zusammensetzung (aus Wasser, organischer Materie und anorganischen Salzen) charakterisiert . . . . Die Hauptmomente dieser Veränderungen sind: a) Abnahme im Gehalte des Organismus an Wasser und flüchtigen Bestandtheilen

von der Entwicklung des Keimes bis zur Höhe des freien Wachs-
thums.''

Davenport's work was followed by a thorough and very labori-
ous investigation by Schaper ('02), who laid particular stress
upon the distinction between intracellular and intercellular growth,
and the relation of the imbibition of water to the intercellular
mode of growth. His results, while including a wider range of
stages, are substantially the same as those of Davenport.
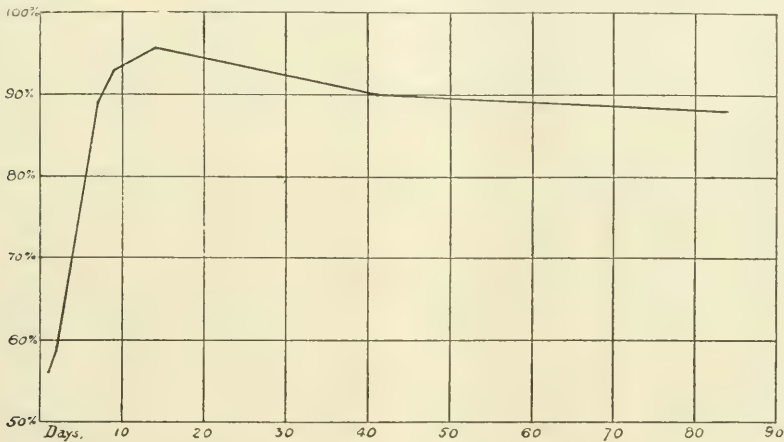
Fig. 4 Graphic representation showing the percentage of water in frog embryos
from one to eighty-four days after hatching. From Davenport, ('97, p. 77, fig. 3)
Proceedings of the Boston Society of Natural History, vol. 28, 1897, by permission
of the Society.

Bialaszewicz ('08), studying this problem quite recently, has
analyzed the water content in frog's eggs not only after hatching
but likewise during the segmentation stages. His conclusion
(p. 819) is that ''das Wachstum der Froschembryonen während
der Entwicklung innerhalb des Dottermembran ausschliesslich
auf der Zunahme der Menge des durch den Organismus aus der
Umgebung aufgenommenen Wassers beruht.'' This investigation
ends the series of experiments which have established the fact that
the frog embryo from the earliest stages of development grows
through imbibition of water from the surrounding medium.

The results of all these observers (Bezold, Davenport, Schaper,
Bialaszewicz), covering, as they do, longer or shorter periods and

obtained more or less systematically, agree with one another very closely, and therefore point strongly to the conclusion that water as a component of the organism plays a very important part in its growth, and furthermore, that its quantity varies according to the intensity of the process of growth, being generally the greatest when growth is at a maximum.

METHOD OF INVESTIGATION

Impressed with the apparent parallelism between formative and regenerative growth, already sufficiently expounded, I have desired to find out to what extent this apparent similarity results from the operation of similar factors. Of the several ways in which the intimate relation between growth and regeneration might be studied, an investigation of the water content at various stages of regeneration seemed the most available and reliable.

My chief reason for choosing the marine polychaet Podarke obscura[1] as the object of these experiments, was my familiarity with its regenerative process gained through previous studies. While presenting in some respects great disadvantages, this worm was preferred to other available animals because the curve of its posterior regeneration had already been worked out in an earlier contribution (Morgulis, '09). The investigation of the water content was conducted in the usual manner. The live animals, first dried thoroughly on filter paper, were carefully weighed; then, having been dessicated over sulphuric acid, they were weighed again. The complete dessication required several days, and the operation was continued until the residue had attained a constant weight. The quantity of water was then easily calculated by subtracting the second weight-determination from the first. All weighings were made by means of a fine chemical balance weighing to one-tenth of a milligram (0.0001 grm.); furthermore, to eliminate possible error due to any inequality in the length of the arms of the balance, the difference method of weighing was employed. This consists in first balancing some constant

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[1] The experiments on Podarke were conducted during the summer of 1909 in the laboratory of the United States Bureau of Fisheries at Woods Hole, Mass.

weight (in the left pan) by the receptacle and the necessary
additional weights. When the object to be weighed has been placed
in the receptacle, the balance is again brought to equilibrium by
removing weights from the right-hand pan. The weights re-
moved equal the weight of the object.

In studying the water content in regeneration one encounters a
very serious difficulty owing to the impracticability of severing
the regenerating tissue from the organism in the early stages; in
later stages, too, when the operation is more or less feasible, the
loss of water by evaporation in the course of manipulating the
organism, and the impossibility of separating accurately the new
from the old tissue would be sources of considerable error in the
final determinations, so that the results could not be very reliable
unless the regenerating organ were of considerable size. All these
difficulties were fully realized in the case of Podarke, a worm
scarcely more than three quarters of an inch in length, where the
regenerated part could be detached and examined by itself
neither immediately after the operation nor even at later periods.
Under the circumstances it became necessary to study the entire
regenerating animals at various intervals after the operation.
Moreover, the animals being too small to be studied separately
with a satisfactory degree of accuracy, it was necessary to examine
rather large numbers of worms at one time, using the average value
for the group as an indicator of the water content at any par-
ticular stage.

I am quite aware that serious objections may be raised against
this procedure, the strength of which no one, perhaps, could feel
more keenly than I myself do. For this reason I shall enumerate
the various objections to which the present investigation is
justly open, and shall then attempt to show that, notwithstanding
the objections, the results still warrant the conclusions.

One of the important defects of any study of this nature is the
lack of a continuous series of results based upon one and the
same group of individuals. It is obvious, of course, that a par-
ticular group of worms could be used to determine the condition
of the water content at only one stage in regeneration,[2] but in

[2] To be sure, other branches of biological research share this disadvantage.

studying different lots of animals as representatives of the differ-
ent stages of regenerations, it must be granted that the continuity
of the process may be traced with a high degree of probability.
In this investigation the stages of regeneration are distinguished
by the number of days elapsed since the operation, and while not
all individuals are in exactly the same conditions, the irregularities
are probably of not much significance, since large numbers of
worms have been used for each determination.

Another serious objection is that the regenerated tissue is not
studied alone, but in conjunction with the old tissue. This, of
course, makes it impossible to decide whether the change in the
water content is localized or not. Fortunately, later examinations
of the quantity of water in the regenerating tails of salamanders,
to which I shall refer again at the close of the paper, show beyond
a doubt that the increase of the water content is here confined
to the regenerating tissue. If the results of this investigation on
salamanders may be relied upon in interpreting phenomena ob-
served in regenerating Podarke, the modification of their per
cent of water may likewise be considered as localized in the regen-
erating tissue. However, as will be seen later, the conclusions
from the study of Podarke are not affected by this complication.

Still another objection to this investigation arises from the
fact that, owing to the smallness of the animal experimented on,
masses of worms, rather than individual worms were studied;
in other words, the results are based entirely upon averages. This
objection is very potent, and in interpreting the facts I have
kept it clearly in mind. The method of averages is certainly open
to many serious criticisms, but the deficiencies of the method may
be compensated to a certain extent by increasing the number of
experiments. For this reason, as will be seen in what follows,
stress is laid only upon phenomena which are fairly constant
in all experiments.

Besides those already recounted, there are several other un-
avoidable sources of error, as might be expected in work which
requires such delicate technique. Every effort, however, has been
exercised to minimize the possibility of error; but in contemplating
the results it will be found necessary to make allowance for
disturbing factors which could not be avoided.

With this exordium, I now reiterate the problem of which this paper is a partial solution. The curves of formative and regenerative growth being strikingly similar the question arises whether both processes are due to similar causes, and especially whether the different stages of regeneration, characterized by different intensities of regenerative energy, are accompanied by corresponding changes in the water content of the tissues, as has been demonstrated to be the case in ordinary growth.

EXPERIMENTS ON PODARKE OBSCURA

All the experiments described below were conducted in the following manner. Several hundred specimens of Podarke obscura, collected in the eel-pond at Woods Hole, were operated upon immediately or very soon after they had been brought into the laboratory. The worms were always cut in two near the middle of the body and the anterior halves were placed in a single large dish half filled with sea-water. In some experiments, the worms were kept in pure sea-water, the water being frequently changed; in others they were also provided with food, consisting of the vegetation of the eel-pond. The outcome of these two sets of experiments, with and without food, though differing in some minor matters, conform to each other in the main. As soon as all worms had been operated on, a sample, taken at random and containing forty to fifty specimens, was examined for its water content. This sample served as control for the experiments, the result being compared with the results of similar determinations made for worms of the same series, but kept for two to forty days after operation before being tested for water content.

First series

Experiment A. This experiment (compare table 1 and fig. 5) was started on July 7, and 40 worms, taken at random out of the 400 operated upon, were examined on that day. These 40 live specimens, previously dried on filter paper to remove all traces of water from the outside, weighed 72.9 mgrms., or on an average 1.823 mgrm. The worms were then dessicated over sulphuric

acid until the dry substance had attained a constant weight (19.1 mgrm.); by subtracting the weight of this residue from the weight of the live animals, it was found that they contained on the average 1.345 mgrm. of water. Expressing the same thing in different terms, these worms contained 73.8 per cent water at the time of the operation. Another sample of 40 worms, also taken at random, was examined two days after the operation, *i.e.*, July 9, and the weight was found to be 67 mgrms., or on an aver-

TABLE 1

GROUP	DATE OF KILLING	DAYS AFTER CUTTING	NUMBER OF WORMS	WEIGHT IN MILLIGRAMS						PERCENTAGE OF	
				LIVE WORMS		DRY SUBSTANCE		WATER		WATER	DRY SUB-STANCE
				Total	Average	Total	Average	Total	Average		
	July										
1	7	0	40	72.9	1.823	19.1	0.478	53.8	1.345	73.80	26.20
2	9	2	40	67.0	1.675	17.0	0.425	50.0	1.250	74.63	25.37
3	12	5	40	65.2	1.630	15.2	0.380	50.0	1.250	76.69	23.31
4	15	8	40	65.2	1.625	14.9	0.373	50.1	1.252	77.08	22.92
5	20	13	40	65.6	1.640	13.5	0.338	52.1	1.303	79.42	20.58
6	25	18	40	50.0	1.250	11.5	0.288	38.5	0.963	77.00	23.00
7	30	23	40	53.2	1.330	12.0	0.300	41.2	1.030	77.44	22.56
	August										
8	9	33	25	29.4	1.176	6.8	0.272	22.6	0.904	76.87	23.13

age 1.675 mgrm. But while the weight of the live worms had decreased in comparison with that of the control group, the relative quantity of water in the body had increased, as is shown by the higher percentage of water—74.63. Five days after the performance of the operation, July 12, a similar group of worms, weighing on an average 1.630 mgrm., was dessicated, and the average water content was found to be 76.69 per cent, or nearly 3 per cent above that of the control. July 15, or eight days after the operation, the per cent of water had increased to 77.08; and five days later still, July 20, thirteen days after operation, the maximum water content (79.42 per cent) was reached. This represents an increase of 5.6. per cent over the control. The per cent of dry substance had meanwhile dropped from 26.20 to a minimum of 20.58.
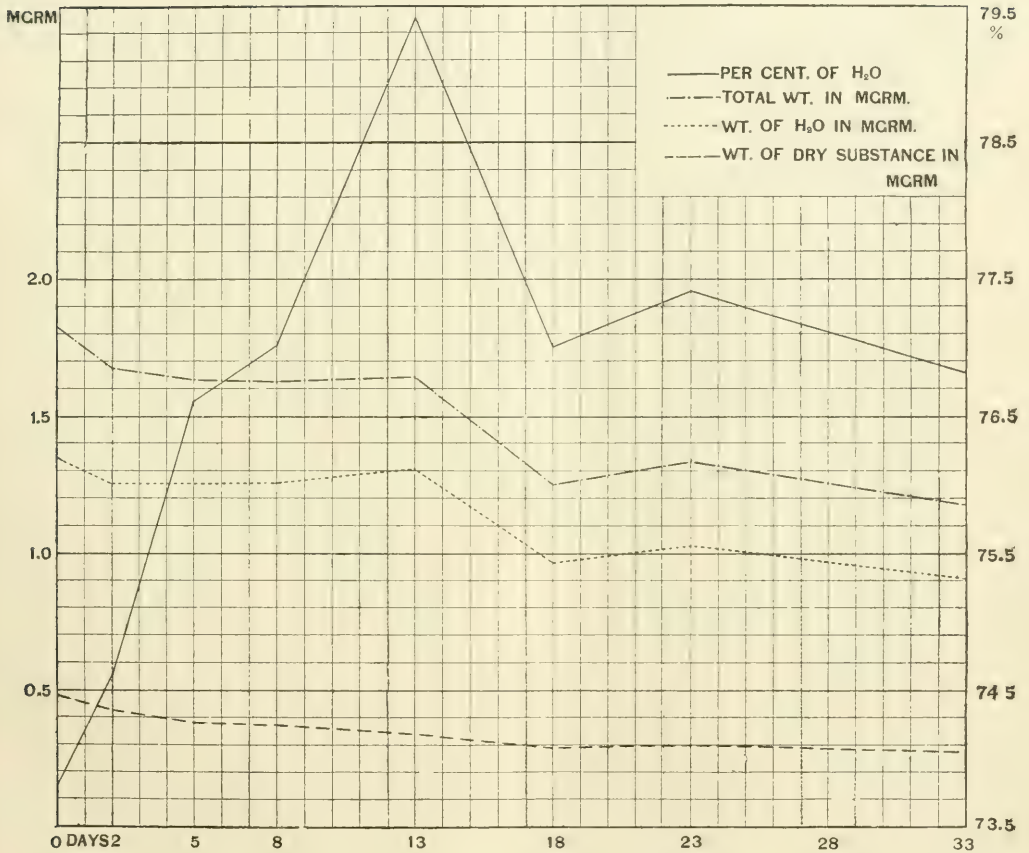
Fig. 5 Curve showing the average weight in milligrams of the anterior half of Podarke obscura at the time of operation and at intervals up to 33 days (dot-and-dash line); of the dry substance (dash line); and of the water (dotted line); also percentage of water (continuous line), based on results recorded in table 1.

Examination of table 1 shows further that the per cent of water content begins to decline after about the thirteenth day. Thus, eighteen days after operation, there was only 77 per cent of water and thirty-three days after operation only 76.9 per cent.

Looking through the average weights of the live specimens, recorded in the sixth column, it will be observed that, disregarding slight fluctuation, the animals were progressively decreasing in weight, having lost about one-third of their weight at the time the experiment ended. The great loss of substance in this experiment is not at all surprising, since the worms were deprived of food; but, as will be seen later, the worms of the second series of experiments were likewise losing in weight, although they were supplied with food. Turning our attention now to the separate constituents of the body, namely, the dry substance and the water, we find that the former suffers proportionally a greater reduction in quantity than the latter. Thus, two days after the operation, the weight of the organism having decreased 8.1 per cent, the dry substance had lost 11.1 per cent while the water had diminished only 6.7 per cent. Again, thirteen days after the operation, when the maximum per cent of water had been attained, the dry substance had diminished 29.3 per cent and the water only 3.2 per cent. And lastly, on August 9, when the per cent of water had declined to 76.87, the loss of dry substance was 43.1 per cent, and that of water 32.8 per cent. Of course, these calculations could be valid only in case the worms of each group contained at the beginning of the experiment exactly as much dry substance and water as the worms of the control group did. Such an assumption is evidently arbitrary; but while the above results are far from being precise, they very probably indicate the nature of the real condition.

The rapid rise of the percentage of water in early stages of regeneration is explicable upon the ground of the proportionately greater loss of dry matter during this period; subsequently, when the loss of water is somewhat accelerated, the percentage content of water diminishes. In this experiment it decreased to 76.87 per cent; but it is probable that had the experiment been carried on for a longer time the per cent would have approached

more closely that which was established for the control, *i.e.*, 73.8 per cent. I do not wish to emphasize these conclusions too strongly at this point, for a survey of the results of the remaining experiments will lend more weight to them. The data can be best appreciated, however, when expressed graphically. Fig 5 contains curves based upon the quantitative determinations recorded in table 1. The horizontal line is divided into equal spaces, each of which represents a period of one day; upon the ordinates are indicated at the left weights in milligrams, at the right percentage of water. The curves show: (1), by a continuous line, the per cent of water; (2), by a dot-and-dash line, the average weight of a live worm; (3), by dashes, that of dry substance, and (4), by dotted line, that of water as determined experimentally for the indicated days. The percentage curve shows for water a rapid rise followed by an abrupt fall; this in turn is followed by a more or less slow decline. It will also be noticed that the period of maximum water content falls within the first fourteen days after operation. The curve bears a general resemblance to similar curves for growing animals.

The other three curves, namely those of the total weight of a worm, of its dry substance and of its water content, all tend downwards. The quantity of dry substance is progressively decreasing, rapidly at first and much slower subsequently. But examining the dotted-line curve, representing the absolute quantity of water in the regenerating worms, it will be observed that, while declining more or less rapidly at the very beginning and during the latter two-thirds of the experimental period, it remains practically at a constant level for some ten days. It should also be observed that the total body weight for the same period is scarcely changed. These facts are especially interesting when correlated with other phenomena characteristic of this period, namely, the greatest intensity of the regenerative process and the highest per cent of water in th he regenerating organism. The last phenomenon is therefore, the resultant of two factors: first, the loss of dry substance; secondly, the retention of water in the organism. Beyond this period the curve of the absolute quantity of water falls off quite rapidly, in fact, more so than the curve showing the quantity
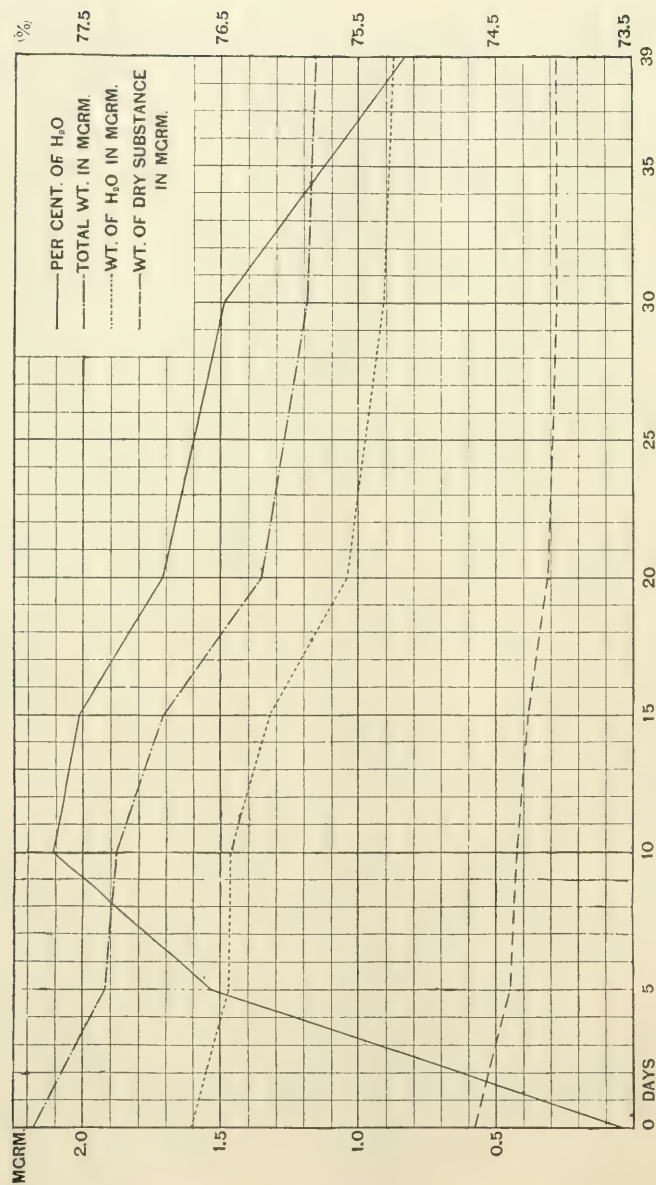
Fig. 6 Curve showing the same features as fig. 5, for the worms of the experiment recorded in table 2

of dry matter, on which depends, of course, the decrease of the percentage of water observed during the subsequent stages.

Experiment B. On July 13, another lot of 400 worms was operated on, of which 50 were immediately picked out at random and weighed. (Compare table 2 and fig. 6.) The average weight of the live worms was found to be 2.180 mgrm., and that of their residue left after their complete dessication 0.576 mgrm., the per cent of the water content being therefore, 73.58. The next deter-

TABLE 2

GROUP	DATE OF KILLING	DAYS AFTER CUTTING	NUMBER OF WORMS	WEIGHT IN MILLIGRAMS						PERCENTAGE OF	
				LIVE WORMS		DRY SUBSTANCE		WATER		WATER	DRY SUBSTANCE
				Total	Average	Total	Average	Total	Average		
	July										
1	13	0	50	109.0	2.180	28.8	0.576	80.2	1.604	73.58	26.42
2	18	5	50	96.0	1.920	22.5	0.450	73.5	1.470	76.56	23.44
3	23	10	50	94.2	1.884	21.0	0.425	73.2	1.464	77.71	22.29
4	28	15	50	85.4	1.708	19.2	0.384	66.2	1.324	77.52	22.48
	August										
5	2	20	50	67.6	1.352	15.6	0.312	52.0	1.040	76.92	23.08
6	12	30	40	47.6	1.190	11.2	0.280	36.4	0.910	76.47	23.53
7	21	39	50	58.0	1.160	14.4	0.288	43.6	0.872	75.17	24.83

mination was made on 50 worms of the same lot five days after the operation. The average weight of a live worm was at this time only 1.920 mgrm., or 11.9 per cent less than in the first or control group. The dry substance suffered a loss of 21.8 per cent, whereas the quantity of water decreased only 8.3 per cent, the result being that the per cent of water increased with remarkable rapidity to 76.56. Ten days after the operation the per cent of water in the regenerating worms had already reached the maximum, 77.71; fifteen days after the operation it had diminished to 77.52, and at later stages fell off more rapidly, being 76.92 twenty days after the operation, and 75.17 thirty-nine days after that event, when the regenerative rate had already been reduced to the lowest limit. The data concerning this experiment are given in

Table 2, and it will be seen on looking through the sixth column
that the animals were continually losing in weight, so that some
six weeks after the operation was performed they had lost 46.8
per cent, or nearly half of the initial mass. The dry substance, as
usual, loses a larger proportion of itself than the water does, but
the difference between the two does not remain the same through-
out the entire regenerative process. This will become clearer upon
inspection of the curves of fig. 6, which shows at a glance the
changes in the weight of the worms, of the dry matter and of the
water, as well as in the percentage of water at different stages
of regeneration. It will be seen from this that the percentage of
water rises to a maximum ten days after the operation, or at the
time of the greatest regenerative energy; then it begins to decline,
tending to return to the normal level. The curve of dry matter
is regularly, and at first very rapidly, sloping downwards, but
in advanced stages of regeneration it remains practically hori-
zontal. The curve showing the quantity of water, however, falls
off more or less rapidly during the later stages when the per cent
of water is likewise diminishing, but remains at a constant level
at the time when the per cent of water in the regenerating
worms is rapidly rising. As was pointed out above, the per-
centage of water increases rapidly in the course of the first
few days because the organism, losing in weight, yields up more
dry substance than water (21.8 per cent and 8.3 per cent, respec-
tively in this experiment). But soon afterwards, since the dry
substance continues to waste away while the water, at least for
a limited time, does not, there is a still further increase in the per-
centage of water.

The two curves of the percentage of water, figs. 5 and 6, are
essentially similar to each other. Both show a rapid increase in
the relative quantity of water when regeneration is most intense,
followed by a continual decrease corresponding in time to the
diminished regenerative activity. Minor differences between these
curves are partly due to the fact that they are based upon an
investigation of differents lots of worms, but more particularly,
as I believe, to the fact that the per cent of water was ascertained
at shorter intervals in the first instance. As to the behavior of

the dry substance and the water in the course of regeneration the curves reveal a very striking agreement.

Experiment C. This experiment was begun on July 13, when 50 of the worms just operated upon were weighed and dessicated. The water content in this group, which served as control for the experiment, was 74.61 per cent. The regenerating worms were then examined at five-day intervals until August 2, and later at ten-day intervals. The results of this experiment was shown in table 3.

TABLE 3

Group	Date of killing	Days after cutting	Number of worms	Weight in milligrams						Percentage of	
				Live worms		Dry substance		Water		Water	Dry substance
				Total	Average	Total	Average	Total	Average		
	July										
1	13	0	50	103.2	2.064	26.2	0.524	77.0	1.540	74.61	25.39
2	18	5	50	89.5	1.790	21.8	0.436	67.7	1.354	75.64	21.01
3	23	10	50	88.0	1.760	20.4	0.408	67.6	1.352	76.82	23.18
4	28	15	50	72.4	1.448	16.8	0.336	55.6	1.112	76.80	23.20
	August										
5	2	20	50	66.6	1.332	15.2	0.304	51.4	1.028	77.18	22.82
6	12	30	40	46.2	1.155	11.0	0.275	35.3	0.882	76.41	23.59

Five days after the operation the water content in the regenerating worms was found to be 75.64 per cent; ten days after the operation, 76.82 per cent; fifteen days after the operation, 76.80 per cent; and twenty days after the operation the water content reached a maximum of 77.18 per cent. Ten days later still the content of water had decreased to 76.41 per cent. The peculiarity of this experiment is that the maximum per cent of water was reached somewhat later than was expected, and indeed later than in any of the other four experiments. Since, however, the determinations of the content of water were made only once every five days, it is quite possible that the highest per cent of water found in this experiment, *viz.*, that observed on the twentieth day, was not in reality the maximum attained during the regenerative process—as this may have occurred some days earlier, but was already a step towards a decrease of the water content. The

increase of the percentage of water shown in this experiment is concomitant with a progressive loss of substance on the part of the regenerating worms, as was also observed in the previous experiments.

Second series

Experiment D. In this series of experiments the proceedure was precisely the same as in the first series, except that food was supplied to the regenerating worms. The experiment was started on August 4, and all the data will be found in table 4.

TABLE 4

GROUP	DATE OF KILLING	DAYS AFTER CUTTING	NUMBER OF WORMS	WEIGHT IN MILLIGRAMS						PERCENTAGE OF	
				LIVE WORMS		DRY SUBSTANCE		WATER		WATER	DRY SUB-STANCE
				Total	Average	Total	Average	Total	Average		
1	August 4	0	50	141.0	2.820	33.8	0.672	107.2	2.144	76.03	23.97
2	4	0	50	135.8	2.716	32.8	0.656	103.0	2.060	75.85	24.15
			Aver.	138.4	2.768	33.3	0.664	105.1	2.102	75.94	24.06
3	9	5	40	98.5	2.458	22.9	0.573	75.6	1.890	76.75	23.25
4	14	10	40	99.6	2.490	21.7	0.543	77.9	1.948	78.21	21.79
5	19	15	40	90.2	2.255	18.8	0.470	71.4	1.785	79.16	20.84
6	24	20	40	93.8	2.345	20.4	0.510	73.4	1.835	78.25	21.75
7	29	25	40	93.4	2.335	20.4	0.510	73.0	1.825	78.16	21.84
8	September 8	35	60	115.2	1.920	26.2	0.437	89.0	1.483	77.26	22.74

As soon as the worms to be studied in this experiment were operated on, two samples, each consisting of 50 specimens, were weighed and dessicated. The per cent of the water content thus determined was 76.03 in the first sample and 75.85 in the second. These determinations are only slightly different, and the average between them—75.94 per cent—will be assumed as the standard, with which the further determinations, made generally every five days, will be compared. It will be seen at once, by looking through the appropriate column in the table, that the per cent of water in the regenerating worms increased steadily up to the fifteenth day, when the maximum of 79.16 per cent was found; after that the per cent of water began to decrease, being 78.25 per cent at twenty
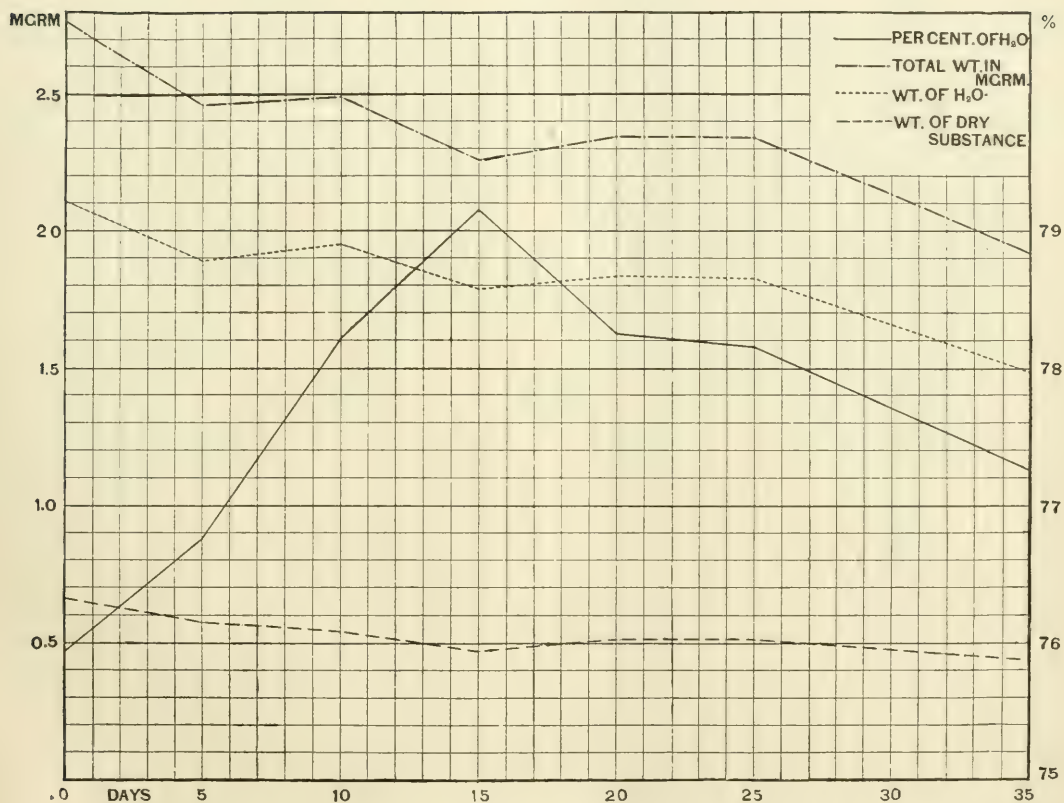
Fig. 7 Curve showing the same features as fig. 5, for the worms of the experiment recorded in table 4.

days, and 77.26 per cent at thirty-five days after operation. Furthermore, an examination of column six shows that the worms gradually lost in weight, though not as much as in the previous experiments. The greatest loss was sustained in the earliest stages (11.2 per cent); at the closing of the experiment the worms weighed 30.6 per cent less than the control specimens. The quantity of dry substance diminishes somewhat during the first half of the experimental period, but remains very nearly constant during the remaining stages. The quantity of water, on the other hand, is lost at a slower rate, and at about the time when the highest per cent of water content is reached it does not decrease at all. In later stages, when the dry substance remains practically constant in quantity, the amount of water diminishes somewhat more rapidly. These points can be best appreciated from the graphic representation (fig. 7) of the data given in table 4.

Considering in their entirety the results of the experiments described above and directing our attention to the essential points in the diagramatic representations of the data, we find that soon after the operation the per cent of water rapidly rises, the maximum usually being reached between the fifth and the fifteenth days after operation. The lack of uniformity in the time at which the maximum per cent of water is observed in the several cases is partly due to reasons indicated in an earlier section of this paper, but partly to the fact that the water content was determined not daily but at intervals of several days. The period of a high percentage of water in the regenerating animals is of short duration, and is followed by a gradual decline in the water content. In short, the changes in the per cent of water during regeneration are expressed by a curve similar to the curve of the rate of regeneration itself, as may be best seen by comparing with one another figs. 3 to 7. The interesting fact is that the period of the highest regenerative rate coincides with that of the highest per centage of water, but whether these phenomena are simply concomitant or whether they stand to each other in the relation of cause and effect is beyond our ken. What concerns us most at this moment is that a similarity exists between the vicissitudes of the water content and the rate of embryonic growth—that the per

cent of water in the tissues increases rapidly when growth is at its height, and diminishes when the latter declines. The parallelism between growth and regeneration, previously discussed on the basis of the periodic change in their intensity, finds further confirmation in the similarity of the curves of the water content in growth and in regeneration.

We are thus led to the generalization that regeneration is the renewal of growth; but in referring regeneration to the category of growth phenomena nothing is gained in the way of a better understanding of its physiology. Growth is one of the fundamental functions in the living world, but its nature is very difficult to analyze, as the multiplicity of definitions it has already received indicates. Growth never occurs apart from other functions and, therefore, cannot be studied entirely by itself; hence these different points of view: that growth is increase in mass, or is continuous change of form, or, finally, is increase in mass accompanied by changes in form. The common meaning attached to growth, however, has made the first definition the more popular. But if regeneration and growth are to be homologized, it may be well to bear in mind that the regenerating organism, at least in the case of Podarke, diminishes in bulk.

These worms were becoming smaller while the new tails were growing and that happened whether or not the animals were fed. It would be of little consequence if the generalization, that regeneration is renewed growth, caused only an inconsistency in definitions; its real significance lies in the implied presence of similar factors in both growth and regeneration. In all studies of embryonic growth it has been shown that at certain stages the body increases through the absorption of water from the surrounding medium. But considering critically the water changes in regeneration it cannot be overlooked that the rise and fall of the per cent of water results from an internal regulation, or a series of occurrences tending to restore normal relations. The increase of the per cent of water here is not due to imbibition of water from the outside, for as a matter of fact the absolute quantity of water in the organism is continually diminishing. It is, furthermore, significant that

during the period of the greatest per cent of water, coinciding approximately with the period of the highest regenerative activity, the organism loses practically no water. This will be better appreciated by consulting diagrams 5 to 7, where the curve of water content forms for a longer or shorter space either a horizontal line or even an ascending one. The retention of water in the organism, resulting in a rapid rise of its relative content, may perhaps have something to do with the properties of the regenerated tissue. Soon after that the quantity of water commences to diminish quite rapidly, so that the per cent of water again approximates the normal.

EXPERIMENTS ON DIEMYCTYLUS VIRIDESCENS[3]

During the winter of 1909-10 I examined the content of water in regenerated tails of the salamander Diemyctylus. The study included only two stages in the regenerative process—at one month and at three months after the operation. The results are, therefore, not sufficient to establish the complete curve of the per cent of water, but in spite of this incompleteness I venture to add these results because of their important bearing upon those previously obtained on Podarke. In the case of Diemyctylus the regenerated tails were severed from the body and separately examined for their content of water, which was then compared with the water content of the old tail. The water content of the regenerating salamander itself was also determined in several instances, and the per cent of water in its tissues was invariably found to be normal. It is, therefore, certain from this investigation on Diemyctylus that the changes in the water content were confined to the regenerated tissue.

From the tables 5 to 9 it can be seen that the quantity of water in the regenerating tails is relatively much greater than in the old tails, and furthermore that it is relatively greater in an early than in a later stage of regeneration. It will be seen on looking through table 9 that, whereas the old tails contained only 74.94 per cent of

[3] These experiments were performed in the Zoölogical Laboratory of Harvard University.

TABLE 5

	I	II	III	IV	V	VI	VII	VIII
	WEIGHT OF TAILS IN GRAMS						PER CENT OF WATER	
DATE	TOTAL		DRY SUBSTANCE		WATER		OLD TAIL	REGEN-ERATED TAIL
	Old	Regen-erated	Old	Regen-erated	Old	Regen-erated		
November 27, 1909 to March 1, 1910	0.3568 0.2849 0.2357	0.1160 0.0543 0.0790	0.2088	0.0180 0.0085 0.0141	0.6686	0.0980 0.0458 0.0649	76.20	84.48 84.16 82.15
Average....	0.2925	0.0831	0.0696	0.0135	0.2229	0.0696	76.20	83.60

TABLE 6

	I	II	III	IV	V	VI	VII	VIII
	WEIGHT OF TAILS IN GRAMS						PER CENT OF WATER	
DATE	TOTAL		DRY SUBSTANCE		WATER		OLD TAIL	REGEN-ERATED TAIL
	Old	Regen-erated	Old	Regen-erated	Old	Regen-erated		
November 30, 1909 to March 5, 1910	0.3656 0.2687 0.3168	0.0690 0.0692 0.1464	0.2202	0.0102 0.0114 0.0258	0.7309	0.0588 0.0578 0.1206	76.85	85.22 83.53 82.38
Average....	0.3170	0.0949	0.0734	0.0175	0.2436	0.0791	76.85	83.71

TABLE 7

	I	II	III	IV	V	VI	VII	VIII
	WEIGHT OF TAILS IN GRAMS						PER CENT OF WATER	
DATE	TOTAL		DRY SUBSTANCE		WATER		OLD TAIL	REGEN-ERATED TAIL
	Old	Regen-erated	Old	Regen-erated	Old	Regen-erated		
November 27, 1909 to March 1, 1910	0.2903 0.2127 0.3005	0.1040 0.0680 0.0722	0.2124	0.0165 0.0102 0.0102	0.5911	0.0875 0.0578 0.0620	73.57	84.13 83.53 85.88
Average.....	0.2678	0.0814	0.0708	0.0123	0.1970	0.0691	73.57	84.51

TABLE 8

	I	II	III	IV	V	VI	VII	VIII
	WEIGHT OF TAILS IN GRAMS						PER CENT OF WATER	
DATE	TOTAL		DRY SUBSTANCE		WATER		OLD TAIL	REGEN-ERATED TAIL
	Old	Regen-erated	Old	Regen-erated	Old	Regen-erated		
November 30, 1909 to March 5, 1910	0.2165 0.2725 0.1960	0.0730 0.0832 0.0925	0.1810	0.0108 0.0126 0.0146	0.5040	0.0622 0.0706 0.0779	73.69	85.21 84.85 84.22
Average.....	0.2283	0.0829	0.0603	0.0127	0.1680	0.0702	73.69	84.76

TABLE 9

	I	II	III	IV	V	VI	VII	VIII
MARCH 1, 1910 TO APRIL 13, 1910	WEIGHT OF TAILS IN GRAMS						PER CENT OF WATER	
	TOTAL		DRY SUBSTANCE		WATER		OLD TAIL	REGEN-ERATED TAIL
	Old	Regen-erated	Old	Regen-erated	Old	Regen-erated		
Average.....	0.2801	0.038	0.0702	0.0026	0.2099	0.0212	74.94	89.08

water (average for four individuals), the tails which had been regenerating in their place for one month and had attained an average length of about 6 mm. contained 89.08 per cent water, or fully 14 per cent of water more than the old tails. From the other four tables (5 to 8) it will be learned further that three months after the operation, when the regenerated tails had already attained an average length of about 15 mm., the average per cent of water varied from 83.6 to 84.76 per cent. The old tails fall within two groups, those with about 73 per cent of water (73.57–73.69) and those with about 76 per cent (76.2–76.85). The increase in the content of water is therefore different for the separate series, but if the average is taken for all four series (5 to 8), it will be found that the regenerated tails three months old contain 7 per cent of water more than the original (amputated) tails (82.08 per cent and 75.08 per cent respectively).

We thus discover that also in the case of the salamander the per cent of water in the regenerating tissue increases rapidly at first (89.08 per cent), then decreases again, tending towards the normal (82.08 per cent). Although these observations are not enough to trace in fullness the changes in the water content of the regenerated tails of Diemyctylus, yet they suffice to substantiate the former results on Podarke as well as to throw light upon some points which remained obscure in those experiments.

SUMMARY

As the curve of formative growth and that of posterior regeneration are essentially alike in their important characteristics, it seemed desirable to investigate the question whether the factors in the two processes are also similar. Numerous experiments on both plants and animals have demonstrated the fact that in formative growth the per cent of water rises to a maximum during the period of rapid growth, and then falls as the organism approaches the adult condition. With this in view, I have studied the water content at successive stages of regeneration in a polychaet—Podarke obscura. The result is practically the same as in formative growth; soon after an operation the water content rapidly rises, reaching a maximum approximately between the first and second weeks; subsequently, it begins to decline. As it was found, furthermore, that the period of maximum water content and the period of maximum regenerative activity approximately coincide, as in formative growth, the similarity between growth and regeneration was thereby shown to be still greater. Close analysis, however, revealed that, while from the point of view of the end result (i.e., the rise and fall of the curve of the per cent of water) growth and regeneration are alike, the two processes involve dissimilar factors. In formative growth the increase in size and in the per cent of water are brought about through imbibition of water from the surrounding medium; in regeneration this does not seem to be the case, as is shown by a comparison of the absolute quantities of water and of dry substance at various stages. The regenerating animals, whether fed or starved, lose in weight

a process which presents three definite phases of regulation of the water content in the organism. First comes a period of rapid loss in weight, when proportionally more dry substance than water is lost, the per cent of water, therefore, increasing. This is followed by a period of rather slow decrease in weight, when practically no water is lost, and when the regenerative activity and the water content both reach a maximum. Lastly there comes a period during which proportionally more water than dry substance is lost, the per cent of water thus declining.

Wien, September 2, 1910.

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EXPERIMENTAL METAPLASIA

1. THE FORMATION OF COLUMNAR CILIATED EPITHELIUM FROM FIBROBLASTS IN PECTEN

G. HAROLD DREW

Beit Memorial Research Fellow

EIGHT FIGURES (THREE PLATES)

CONTENTS

INTRODUCTION AND REVIEW

The experiments described in this paper were performed on Pecten maximus and Pecten opercularis at the Plymouth Laboratory of the Marine Biological Association of the United Kingdom.

In the course of some investigations as to the mode of formation of new fibrous tissue in Pecten, undertaken by Mr. W. De Morgan and myself (Drew and De Morgan '10) we transplanted various tissues, such as the digestive gland, gills, gonad, etc., into the middle of the adductor muscle, and studied the process of fibrous tissue formation around the implanted mass. We also injected sterile Agar jelly made with sea water, and since then I have performed similar experiments with sterilised cotton wool, cork, elder

pith and other foreign bodies which are presumably unaffected by the body fluids of Pecten. In these experiments it was noticed that the reaction of the tissues to the implantation of portions of the ovary presented, in the later stages, marked differences from the reaction to any of the other tissues or foreign bodies employed, and it was with the object of explaining these differences that the work here described was undertaken.

Briefly summarised, my results show that after implantation of a portion of the ripe ovary into the adductor muscle, a layer of fibroblasts is formed around it, and coincidently the ovarian tissue is invaded by phagocytes and degenerates. After the lapse of about six days no trace of organised ovarian tissue remains, but there is left a cyst surrounded by fibroblasts, and containing blood corpuscles and a quantity of small granules having the orange color of the yolk substance. After the lapse of about 20 days more, the innermost fibroblasts gradually change their shape, and form a layer resembling columnar epithelial cells, which later become ciliated. Eventually the whole cyst becomes lined with well defined ciliated epithelium, which persists at least for 120 days, which is the longest period I have yet succeeded in keeping the animals alive in the experimental tanks. I consider that there is some evidence to show that this change of the fibroblasts into ciliated epithelium is a reaction to the presence of some definite chemical substance within the cyst.

In the vertebrate many cases of the change of a tissue of one type into another type are known to occur, and to such changes the name Metaplasia has been given. According to Adami, ('08) they have this in common, that "Epithelial (epiblastic and hypoblastic) tissues can only be converted into other forms of epithelial tissue, and one form of mesoblastic into another form of mesoblastic."

Such examples as the conversion of cartilage into bone, or of connective tissue cells into fat, are cases of physiological metaplasia of mesoblastic tissues, and it is to be noted that these changes occur in the adult and not only in the course of development. Instances of pathological metaplasia are numerous. Thus among the epithelial tissues we have the change of the columnar ciliated

epithelium of the larynx into a squamous type as the result of chronic irritation, the conversion of the pavement epithelium of the bladder into the columnar type found in papillomatous overgrowths, and a similar change in cases of ectopia vesicae where even glandular crypts of a very simple type may appear.

Of special interest are the experiments of Wolff ('93) on the eye of the larval newt and salamander. In these animals, if the lens be removed, it is regenerated from the iris, and it has also been shown that the retina itself can, under certain conditions, produce bodies resembling lenses. Here we have a case of metaplasia between totally dissimilar tissues, differing greatly in their mode of formation, but which still are both of epiblastic origin. A somewhat similar case is that described by Saxer ('04) who found a tissue resembling columnar epithelium lining the cysts that are of relatively common occurrence in gliomata.

Among the mesoblastic tissues there are many common instances of metaplasia. For example, the ossification of tendons and muscles, the formation of true bone in the lungs, in old pleural and pericardial adhesions, and in the fibroid valve of chronic endocarditis. Harvey ('07) has experimentally caused the formation of bone in the walls of arteries of rabbits by injury, and a similar formation is often found in cases of arterio-sclerosis of long standing.

Examples of the change of tissue derived from one germ layer into closely allied tissues derived from the same germ layer might be multiplied indefinitely, but it must be noted that there are many pathologists who deny this frequent occurrence of metaplasia, and explain these changes in other ways. Foremost of these is Ribbert. He considers, for example, that the replacement of columnar by squamous epithelium is due to the overgrowth of included islands of squamous cells, under altered conditions of environment, and he has other explanations for most of the cases here cited. His conclusions, as summarized by Adami ('08) are to the effect that "only tissues that, while externally different, possess nevertheless the same histogenetic capacities, can undergo metaplasia, one into the other." On the other hand, Leo Loeb ('99) has recorded that in cases of epithelial regeneration

in vertebrates, he has observed epithelial cells migrate into the underlying tissues, and take on the appearance of fibroblasts.

Much of the work that has been done on regeneration is of interest in this respect. Braun ('03) has pointed out that in regeneration in tadpoles, epithelium may give rise to nerve tissue. There is the work of Barfurth('91-'00) and Fraisse('85)on regeneration of the tail in several Urodela, and in the tadpole of the frog, and of Towle on the limbs of Plethedon. These observers find that each tissue only reproduces tissue similar to itself, but their experiments were necessarily confined to types in which regeneration occurs, and dealt only with the phenomena consequent on amputations. Much more work has been done on the regeneration of single tissues or organs, and in these cases it has been found that like always reproduces like, and that metaplasia does not occur.

To turn now to invertebrates, we have the observations of Miss Reed, recorded by Morgan, ('04) on the regeneration of the claw of the crayfish and hermit crab, in which it is conclusively shown that the whole claw with its muscles, etc., is regenerated from the ectoderm, i.e. metaplasia of ectodermal into mesodermal cells occurs. Again there are the experiments of Koeber ('00) on the regeneration of the pharynx of Allolobophora, in which he shows that though the epithelium lining the pharynx is developed from the ectoderm, yet in the course of regeneration it grows from the endoderm continuous with that lining the alimentary canal.

Flexner ('98) has pointed out that in regenerating Planarians the surface epithelium may give rise to distinct nerve elements, and in this case, though both the surface epithelium and the nerve tissue are originally derived from the same germ layer, yet we have metaplasia occurring between tissues which are histologically totally different, and indeed the process may in some ways be considered analogous to the regeneration of the lens from the iris in the eye of the larval newt and salamander described by Wolff ('94).

It thus appears that, whatever may be the case in vertebrates, in lower types of animals there is much evidence to show that metaplasia may occur between cells derived from different germ layers, and between cells possessing widely different histological

characters. With regard to the cytology of the changes involved in regeneration and metaplasia, Kölliker ('85) considered that regeneration of an organ or tissue cannot occur unless that organ or tissue contains cells of an embryonic character, or at least contains elements that are able to assume embryonic characters. The question has been more fully dealt with by Adami ('00), who comes to the conclusion that the fully differentiated cells of a tissue proper never arise from cells that are themselves fully differentiated, but during physiological regeneration arise from certain 'mother-cells', which are normally present in the tissues. He considers that "under abnormal conditions, the fully differentiated functioning cells of certain tissues are capable of proliferation and giving rise to cells of like nature, but this is only after a preliminary reversion to a simpler, more embryonic type."

It will be shown in the present paper that under the experimental conditions, the fibroblasts of Pecten revert to a somewhat undifferentiated embryonic type, and then become converted into columnar ciliated epithelium.

DESCRIPTION OF TISSUES INVOLVED IN THE EXPERIMENTS

An excellent and detailed account of the anatomy of Pecten maximus and Pecten opercularis is given by Dakin ('09) in his monograph on Pecten. The two species are very closely allied, and except in point of size, resemble each other both anatomically and physiologically to a remarkable degree.

The adductor muscle consists of two portions, bound together by the same sheath of connective tissue, but differing in structure. The larger, white and semi-transparent, consists of striated fibres. The fibres of the smaller, which is of an opaque and dead white appearance, and lies against the posterior surface of the larger mass, are non-striated. It was into the larger mass that all material in these experiments was introduced. Superficially the muscle is covered with a layer of columnar epithelium continuous with that lining the mantle.

There is a large blood supply to the muscle from the adductor artery (Dakin), and it contains numerous lacunar spaces. Scat-

tered through it are numerous strands of connective tissue. These contain fibroblasts with very elongated, deep staining nuclei, and long fibrillar processes.

The gonad consists of a semicrescentic mass attached at its base to the adductor muscle. When ripe the male proximal portion is creamy white in color, and the distal female part is of an orange or vermilion hue; the boundary between the male and female portions is sharply defined.

In Pecten maximus the loop of the intestine reaches almost to the apex of the ovary, whilst in Pecten opecularis it does not extend much beyond the testis.

Microscopically the gonad consists of branched tubules, terminating in alveoli lined with germinal epithelium: when ripe the alveoli are crowded and distended with ova or sperm, and it is not always easy to trace the connecting tubules. These tubules join up to form two main ducts which are lined by columnar ciliated epithelial cells, the height of which is about twice the width while the cilia are about as long as the cells (Dakin). Traced towards the alveoli, the cells lining these ducts become shorter and lose their cilia, and in the smallest tubules are of a flattened almost squamous type, where finally they appear as if they were directly continuous with the germinal epithelium.

The ripe ova are of an orange or vermilion color, measuring about 50 μ in diameter, the nucleus is relatively large, the nucleolus conspicuous, and the cytoplasm crowded with yolk granules. The spermatozoa are small and of the typical shape, with a long flagellum attached to the broad end.

The blood of Pecten is a slightly cloudy, colorless fluid, it does not coagulate, but when shaken a number of small, white, floccular masses appear, which soon fall to the bottom of the tube leaving the supernatant fluid clear and transparent. These masses consist of blood corpuscles, agglutinated to form plasmodia.

The corpuscles although varying in size, are only of one kind. They are amœboid bodies, which when expanded protrude a number of slender pseudopodia. When contracted, they are ovoid or spherical. There is a single compact nucleus, staining

readily with methylene blue. The cytoplasm is finely granular, and stains with eosin, but there are no large eosinophil granules. According to Cuenot,('91) they originate in a 'glande lymphatique' situated at the base of the gills.

In a former paper (Drew, '10) I have shown in the case of Cardium norvegicum, that when the corpuscles come in contact with a rough foreign body, or with injured tissue, they possess the power of agglutinating and forming a compact plasmodial mass. In this way bleeding from a small wound is stopped. When the edges of a wound are covered with this mass of agglutinated corpuscles, proptoplasmic strands are formed across the wound, connecting the plasmodia, these strands thicken and contract, and so approximate the edges of the wound. I have repeated these observations on Pecten, and find that the same phenomena occur, and that a similar plasmodial mass of agglutinated corpuscles is rapidly formed around any tissue implanted into the adductor muscle.

That Lamellibranch blood corpuscles are capable of a phagocytic action towards degenerated cells has been shown by De Bruyne ('96) in the case of Mytilus edulis, Ostrea edulis, Unio pictorum and Anodonta cygnea. Sir Ray Lankester ('86 and '93) has shown that certain corpuscles of Ostrea edulis have a phagocytic action on diatoms and minute green algae, and I have shown (Drew, '10) that the corpuscles of Cardium norvegicum have a phagocytic action on bacteria, and are attracted towards extracts of dead tissues. It has also been shown that the corpuscles of Pecten maximus exercise a similar phagocytic action on dead cells (Drew and De Morgan '10).

The formation of fibrous tissue around a foreign body implanted into the adductor muscle has been described in a former paper (Drew and De Morgan '10). The normal fibroblast is an elongated cell, with a spindle shaped nucleus and an indefinite amount of cytoplasm which appears to be drawn out and connected with the neighboring cells by fine fibrillar processes. These cells are usually connected with each other by slender strands of some collaginous substance, which forms the groundwork of the fibrous tissue, and in the normal resting stage it is often impossible to dis-

tinguish between these collaginous strands and the fibrillar pro-
cesses of the cytoplasm.   When about to divide amitotically,
the fibroblasts become shorter and thicker, the cytoplasm van-
ishes, and an oval or round nucleus with a reticulated arrangement
of the chromatin results; eventually this splits in two and the two
halves separate.   The process is shown in fig. 1.

The details of fibrous tissue formation differ in the early stages
according to the degree of irritative action of the foreign body, and
the consequent amount of inflammation produced.   If the inflam-
mation is very slight, as was the case in most of the experiments
about to be described, the implanted body is first surrounded by a
thin layer of agglutinated blood corpuscles.   This is followed by
the rapid amitotic division of the fibroblasts in the neighborhood,
they lose the typical spindle shape of their nuclei, and the new
formed cells consist of rounded or oval nuclei with a scarcely per-
ceptible amount of cytoplasm.   These rounded cells migrate
towards the implanted body, and arrange themselves in layers
around it, the nuclei become elongated, and the proportion of
cytoplasm increases.   Finally a cyst wall of typical fibrous tis-
sue is formed, surrounding and completely shutting off the im-
planted body.

METHODS

Both Pecten maximus and Pecten opercularis can be obtained
in large numbers by dredging in the neighborhood of Plymouth.
It was found necessary to allow these animals to become accli-
matised to living in the laboratory tanks before proceeding to the
experimental work.   When first placed in the tanks, the mortal-
ity was heavy, often amounting to 30 per cent in the first three
days, but after the lapse of about a week the survivors appeared
to be fully acclimatised to the changed conditions, and often re-
mained healthy for some months.

Experiments on animals whose health was doubtful were of
no value, both because the shock consequent on the injection of
the foreign body frequently caused death, and also because the
reaction of the tissues was not normal in unhealthy specimens.
When a Pecten is healthy, it lies with the valves of the shell

slightly apart, the tentacles are expanded, and it responds rap-
idly to any stimulus by closing the shell; when held up in the air
the water which drains away is clear and contains no slime. An
unhealthy specimen lies with the valves of the shell wide open,
there is little or no response to stimuli, and the valves only close
under pressure. The tentacles are retracted, and the gonads,
gills, and tissues generally look flabby and unhealthy. The water
which flows out between the valves is slimy and viscid, and this
is generally the first sign of deterioration.

When making an implantation of the ovary, a healthy speci-
men was chosen in which the gonad was of a full orange or ver-
milion color, and obviously distended with ova. The valves
of the shell were wedged apart with a cork, and the interior well
washed with a brisk stream of sterile sea water from a wash bottle
that had previously been steamed for some time in a 'Koch.'
The adductor muscle was then severed with a scalpel, and one
valve of the shell turned back. The extremity of the ovary was
cut off, and while held by forceps, was very thoroughly washed
with a stream of sterile sea water, then it was placed in a steril-
ised Petri dish containing a little sterile sea water, and kept care-
fully covered.

All instruments were sterilised by boiling in dilute caustic soda
solution, and were washed in sterile sea water to remove any trace
of the caustic soda immediately before use. They were always
re-sterilised between each experiment. When not in use it was
found more convenient to keep all steel instruments in lime water
instead of drying them, as they are particularly liable to rust
after being exposed to the action of sea water.

The transplanting needles, made of a platinum and iridium
alloy, resemble large hypodermic needles. Two sizes were used,
the larger for experiments on P. maximus, measured about 6
cm. in length, and 1 mm. in diameter, the smaller for experiments
on P. opercularis was of the same length, but about half that
diameter. Into the hollow needle a somewhat longer stilet fits
closely, and works like a piston. Any material taken up in the
point of the needle is sucked in by drawing the stilet back, and
again ejected by pushing it forward. Small portions of the ovary

were cut off by fine scissors and drawn up into the needles in this
way.

In the earlier experiments portions of the ovary were injected
into the muscle through a hole bored in the shell.   The holes were
drilled in the convex or right valve by an ordinary dentist's drill,
the head of which was prevented from penetrating too deep by a
lapping of thread.   The spot selected for drilling was sterilised
with a saturated solution of corrosive sublimate, washed off with
a solution of hydrogen peroxide (20 vols.) or distilled water, care
being taken not to allow any of the sublimate to run between the
valves.   The transplanting needle was then introduced to the
required depth, slightly withdrawn and its charge projected into
the channel.   The hole was then thoroughly dried, and stopped
with sealing wax.   In later experiments it was found that this
proceeding, which occupies a good deal of time, was unnecessary,
and the implantation was made directly into the muscle from the
side.   By this method there is a slightly greater risk of sepsis
and the consequent death of the animal, but this is more than com-
pensated for by the saving of time which it entails, and this is of
importance when a large number of experiments have to be made.
In some cases much larger pieces of the ovary were implanted
by simply making a longitudinal slit in the muscle with a small
scalpel, and inserting the ovarian tissue with fine pointed forceps.
The wound thus made is closed by the contraction of the muscle,
but the risk of sepsis when this method is employed is considerable,
and only a small proportion of the animals survived the experi-
ment long.

During the experiments the animals were kept in the laboratory
tanks, or in basins with a continuous flow of water.   Exposure
to too strong a light should be avoided, and if the animals are
required to live long it seems to be of advantage to cover the basins
with green glass, or to moderate the light in some other way.

When required for examination, the animals were killed by wedg-
ing the valves of the shell apart with a cork, and then placing in
dilute spirit.   When dead, one valve of the shell was removed,
and the adductor muscle carefully sliced with a razor until the
orange color of the implanted ovarian tissue could be seen shining

through the semi-transparent muscle. A small cube of the muscle containing the ovarian tissue in the middle was cut out and placed for about three hours in Zenker's fluid, well washed, treated with dilute iodine, and finally embedded in paraffin. Serial sections were then cut, and stained in very dilute Delafield's hæmatoxylin. Other stains and fixatives were used for especial purposes, but the above procedure was found to be the most satisfactory as a routine method.

It is noteworthy that even after the ovarian tissue has been implanted into the muscle for as long as four months, the orange color is not lost or even diminished in intensity, so that the site of the implantation can always easily be distinguished.

RESULTS OF THE IMPLANTATION OF PIECES OF THE MATURE OVARY INTO THE ADDUCTOR MUSCLE, AND THE SUBSEQUENT DEVELOPMENT OF CILIATED EPITHELIUM FROM THE FIBRO BLASTS FORMED AROUND IT

The sequence of events after the implantation of pieces of the ripe gonad of one specimen of Pecten maximus into the adductor muscle of other animals of the same species is identical with that occurring when the same experiments are performed on Pecten opercularis.

Pieces of the ripe ovary after ejection from the transplanting needle into the muscle, are roughly spherical in shape, and measure from 1 mm. to 0.5 mm. in diameter according to the size of the needle used. Very soon after implantation such a piece of ovarian tissue becomes surrounded by a thin layer of agglutinated blood corpuscles, and the track of the needle is closed by a similar mass of blood corpuscles forming a plasmodial mass, which, by its contraction, draws together the tissues that have been displaced by the passage of the needle (Drew, '10). This condition can be seen in sections from animals that have been killed about one hour after the implantation has been made. If the operation has been conducted aseptically, the resulting inflammatory reaction is very slight. There appears to be a definite determination of the blood cells towards the ovarian tissue, but there is nothing approaching

the condition of venous engorgement and stasis that occurs when a septic tissue is implanted (Drew and De Morgan '10).

After a time fresh blood corpuscles penetrate the thin agglutinated layer, and start a phagocytic action on the ovarian tissue. Meanwhile the fibroblasts in the walls of the blood spaces, and in the intermuscular connective tissue in the neighborhood, undergo division. This division is amitotic, and commences about twelve hours after the implantation. Before division the fibroblasts lose their spindle shape and become oval: a split then appears at one end, and progresses in the plane of the long axis of the nucleus until two daughter nuclei are formed, attached to each other at one extremity, and inclined at an acute angle to one another. These gradually straighten out until they form an hour glass shaped mass of nuclear material. Finally the two nuclei are separated at the constriction, and two oval or circular cells are produced, having large nuclei with relatively very little cytoplasm, and bearing no resemblance to the spindle shape of a resting fibroblast.

There follows a migration of these cells with round and oval nuclei towards the site of the implantation. They chiefly follow the course of the strands of fibrous tissue bounding the blood spaces, but many migrate in all directions between the muscular fibres.

On reaching the layer of agglutinated corpuscles surrounding the implanted tissue, the fibroblasts arrange themselves in rows; and their nuclei elongate in such a direction that their long axes form arcs of a circle surrounding the implanted ovary.

This surrounding layer presents a somewhat stratified appearance. At first it contains a number of blood-corpuscles, but these eventually are removed, probably by autolysis, leaving only the fibroblasts.

In these experiments the layer of fibrous tissue formed in this way was always very slight, usually not more than two or three cells thick. If by any error sepsis occurred, it was followed by a violent inflammatory reaction, and if the animal survived, by subsequent great formation of fibrous tissue.

Meanwhile the implanted ovarian tissue shows signs of degen-

eration. The cells of the germinal epithelium, the connective tissue cells, and the cells lining the oviduct, lose their normal appearance, the chromatin of the nuclei becomes aggregated into small darkly staining masses, and the outline of the cells becomes less distinct, the cilia of the oviducal epithelium soon vanish. Coincidently the whole mass of tissue is invaded by blood corpuscles which exercise a phagocytic action and slowly remove the degenerated material. The mature, or nearly mature, ova show a much greater resistance to this degenerative process than any of the other cells.

Thus, after the lapse of about three days from the implantation, we have a mass of ovarian tissue which is invaded by phagocytes and shows signs of degeneration, and is surrounded by a layer of fibroblasts forming a definite cyst wall. The fibroblasts are mostly oval in shape, with little or no perceptible amount of cytoplasm, and have not taken on the appearance they present in the resting state.

From the fourth to the sixth day degeneration of the ovary continues, and when cyst formation takes place, as in the cases here described, the degeneration is complete and every trace of organised structure has vanished by the sixth day. When the degenerative changes are complete, the site of the implanted ovarian tissue is occupied only by blood cells, and by a granular substance, which must either be formed during the process of degeneration, or be left after all the other substances composing the ovarian tissue have been rendered soluble and so have escaped from the cyst. If the cyst is cut open, and the contents examined under the microscope, it is seen that the granular matter is of an orange color, and that many of the blood corpuscles have ingested particles of this substance. As the orange color of the cysts remains unchanged, and undiminished in intensity, even after four months, it appears that this substance is unable to escape from the cyst through the surrounding layer of fibrous tissue, and the same must hold true for blood corpuscles within the cyst, which have absorbed this substance by phagocytosis. It seems probable that such blood corpuscles, after ingesting granules of this substance,

degenerate, and eventually become dissolved, leaving the granules behind.

Fig. 2 shows the condition at the fifth day. The degeneration of the ovarian tissue is practically complete, though traces of the ova still remain: this tissue is surrounded by layers of oval fibroblasts, and the fibroblasts in the neighborhood are still dividing and migrating towards the cyst wall. Blood corpuscles are making their way into the cyst between the fibroblasts forming its wall.

The subsequent changes occur more slowly. The fibroblasts forming the innermost layer of the cyst wall remain unchanged for some time, but those forming the outer layers regain the typical elongated spindle shape of the resting fibroblast. Then, after a period varying from eighteen to twenty-five days from the implantation, the fibroblasts in immediate proximity to the degenerated ovarian tissue alter their appearance. The nuclei become rounder and the cytoplasm of each joins up with its neighbor, forming a faintly staining and somewhat indefinite continuous layer (fig. 3). Sections of a little later date show this layer more defined and also a change in the character of the nuclei. An aggregation of the chromatin resembling a nucleolus appears, and from this thin strands of chromatin radiate to the periphery. No cell walls are visible between the nuclei, which appear to have reverted to an embryonic type. Fig. 4 shows this condition, the contents of the cyst are surrounded by a continuous layer of nuclei with definite nucleoli, and these nuclei are embedded in a mass of cytoplasm having no dividing cell walls.

In the course of a few days these nuclei again alter in shape (fig. 5), they become smaller and more oval, and the nucleoli disappear. The surrounding cytoplasm becomes more definite, and stains deeper, and a distinct boundary or basement membrane appears between it and the layers of fibroblasts.

Shortly after this, long slender cilia appear from the inner border of this layer of cells (fig. 6). These cilia are very delicate and at first irregular in length, varying from a length about equivalent to the depth of the cells to more than twice that amount. The date of the appearance of cilia varied from 21 to 32 days, and seems to have some relation to the amount of ovarian tissue

implanted. Thus it certainly occurred earlier when large cysts, measuring about 4 mm. in diameter, were produced by cutting the muscle and inserting large pieces of the ovary, than when a transplanting needle was used.

In the course of time, lateral walls dividing the cells appear, and these are usually clearly visible about 40 days after the implantation. Thus eventually there is formed a closed cyst, the wall of which is lined with typical columnar ciliated epithelium and surrounds a mass of orange-colored granular debris, in which are numerous blood corpuscles in varying stages of degeneration (figs. 7 and 8). This cyst remains unaltered for at least 120 days, which is the longest period during which I have succeeded in keeping the animals alive under experimental conditions.

So far the sequence of events after implantation of pieces of the ovary producing subsequent cyst formation have been described, it will now be necessary to enter into the modifications of this process that occur when cyst formation does not take place.

If extremely small pieces of the ovary are implanted, or if, as sometimes happened in the experiments, the ovarian tissue was distributed thinly all along the track of the needle, cyst formation around the whole implanted mass does not occur. In such cases there is at first a very slight formation of a surrounding layer of agglutinated blood corpuscles, but beyond this, if the experiment has been carried out aseptically, there is very little reaction of the tissues to the implantation in the first few days. The implanted tissue often appears quite normal for as long as a week, or eight days, but after this, degenerative changes set in, and by the thirteenth day at latest, all trace of life in the cells of the oviduct, germinal epithelium, or ova has vanished. Meanwhile the degenerating tissue is invaded by blood corpuscles and fibroblasts. The latter tend especially to invade and travel along any remaining framework of connective tissue that may be left in the implanted mass, and thus to form a dense mass of new fibrous tissue in the place of the old. From the examination of a large number of sections of different stages of this process I am of the opinion that all implanted fibroblasts die, and that all the new fibrous tissue is derived from the cells of the host, though the matter would be extremely difficult to prove definitely.

As the actual disintegration of the ripe ova in the course of the degenerative changes occurs very slowly, the invasion of fibroblasts causes the ova to become completely surrounded by new fibrous tissue. Thus are produced a number of minute cysts containing very few ova, or possibly only one. Subsequently these cysts become lined with columnar ciliated epithelium, derived from the innermost layer of fibroblasts forming the cyst wall, in the manner already described.

It often happens, even when the whole implanted mass becomes encysted, that small aggregations of ova near the outside form separate cysts, and also develop a lining layer of ciliated epithelium. Similarly when small pieces of ovary are implanted, the fibroblasts, by traveling along any connective tissue framework present in the ovary, may divide the whole cyst into several partitions separated by thin layers of fibrous tissue, which subsequently also form ciliated epithelium.

EXPERIMENTS TO DETERMINE THE RELATION OF THE PRESENCE OF OVARIAN TISSUE TO THE FORMATION OF CILIATED EPITHELIUM FROM FIBROBLASTS

An attempt was made to cause the formation of ciliated epithelium by the implantation of portions of the ovary that had been killed in various ways, but all these were unsuccessful. The following methods were tried:

1. *Heat.* Small portions of the ovary were boiled in sea water for various times ranging from 2 minutes to 15 seconds, and then implanted in the muscle in the usual way. A considerable inflammatory reaction resulted, followed by extensive formation of fibrous tissue: a very large proportion of the animals died within a week of the implantations, and often the track of the needle did not heal up. In none of these experiments did the animals survive for more than 15 days, and in all these there was an opening leading to the surface from the implanted tissue.

Pieces of the ovary that had been heated for two minutes to 70°, 60,° 50,° and 45° C. before implantation gave similar results, but heating to 40° C. for two minutes in some cases did not affect

the ovarian tissue, that is to say, cysts lined with ciliated epithelium were produced in a small proportion of these experiments. The implantation of pieces of ovary that have been heated to 30° C. produces the same results as the implantation of unheated portions.

2. *Cold.* Small pieces of the ovary were frozen (at a temperature of −20° C.), and allowed to thaw slowly. The thawing process took about an hour, then after the lapse of 15 minutes the ovary was again frozen. This process was repeated four times, and then the pieces of ovary were implanted in the usual manner. The animals were killed after 35 days, and sections showed that a moderately intense inflammatory reaction had resulted; the implanted tissue was invaded and nearly completely replaced by blood cells, and was surrounded by a thick and compact layer of fibroblasts. No ciliated epithelium was present, nor did the inner layer of fibroblasts show any of the changes preliminary to its production.

3. *Chemicals.* Pieces of the ovary were treated for one hour with various protoplasmic poisons, then well washed in sterile sea water, and implanted. Solutions of the following substances were tried:

> 1 part corrosive sublimate in 2000 parts of sea water.
> 2 per cent phenol in sea water.
> 2 per cent of 30 per cent formalin in sea water.
> 1 part of 20 vols. hydrogen peroxide to 10 parts of sea water.
> 0.5 per cent of potassium cyanide in sea water.
> 20 per cent alcohol in sea water.
> 0.5 per cent of chloroform in sea water.

In every case the animals died within ten days of the experiment, showing signs of intense inflammation and often liquefaction of the tissues at the site of the implantation.

4. *Degeneration in vitro.* The ovary was thoroughly washed in sterile sea water, cut in small pieces, and each piece placed in a sterile test tube with a little sterile sea water. Similar pieces were placed in test tubes containing the blood of Pecten, collected under aseptic conditions. The ovarian tissue was allowed to degenerate in these tubes for three days, then cultures were made

with a loopful of the fluid from each tube on sloped fish broth peptone gelatin, made with sea water, and the pieces of the ovary were implanted into the muscle in the ordinary way. It was found, considering only those cases which the cultures showed to be sterile, that about the same proportion of the animals survived as in check experiments, but whether the degeneration had occurred in blood or in sea water, in no case was there any production of ciliated epithelium.

Experiments in which the ova were removed from the ovary and then injected were unsuccessful. If the ova be shaken out into sterile sea water, centrifugalised, or allowed to settle, and then injected with a hypodermic syringe, it was found that a large proportion of the animals died within a week, and in those that survived it was impossible to find the ova on dissection. To obviate this difficulty the centrifugalised ova were placed in a sterilised solution of gelatin in sea water, at a temperature just above the point of solidification, this was allowed to cool, and portions of the jelly implanted into the muscle: unfortunately this always resulted in the rapid death of the animal, presumably because the gelatin has some toxic action. Agar jelly could not be used for this purpose because it solidifies at too high a temperature, and also is not dissolved by the body fluids of Pecten.

A number of experiments were performed to prove that the development of the lining layer of ciliated epithelium of the cysts was not produced merely as a result of irritation, and as a reaction to the implantation of any foreign body. In a previous paper (Drew and De Morgan '10) the result of the implantation of the tissue forming the gills and digestive gland of Pecten, and of sterile Agar jelly, has been studied. In addition to these, such substances as sterilised cotton wool, cork, elder pith, and small portions of sterilised silicious sponges were implanted to act as a source of irritation. In all these cases the formation of a cyst wall composed of fibrous tissue took place, but there was no development of ciliated epithelium, and the same holds good for cases where the organ of Bojanus of Pecten maximus was implanted into other animals of the same species.

The transplantation of the ovarian tissue of animals of different

species into Pecten maximus or Pecten opercularis in every case caused death in a very short time. Such experiments were tried with the ovary of Cardium edule, Cardium norvegicum, Glycimeris glycimeris, and various species of Tapes and Venus. On the other hand, after the transplantation of the ovary of Pecten opercularis into the muscle of Pecten maximus and *vice versa*, the animals survived indefinitely, but there was no development of ciliated epithelium within the cysts formed round the ovarian tissue.

The implantation of portions of the male gonad produced a violent inflammatory reaction, with subsequent very extensive formation of fibrous tissue for some distance around the site of implantation. No cyst formation took place, and no trace of the spermatozoa could be seen in sections made three days after the experiment. The implantation of large pieces of the testis (more than about 1 mm. in diameter) usually caused the death of the animal in three or four days.

If small pieces of the ripe ovary be placed in sterile sea water containing a little sperm, then well washed and implanted, a similar inflammatory reaction and free formation of fibrous tissue results, only a small proportion of the animals survive, and in these no formation of ciliated epithelium occurred. Similarly, if some days after implantation of pieces of the ovary, a little sperm suspended in sterile sea water be injected with a hypodermic syringe into the exact site of the original implantation, inflammation and free fibrous tissue formation is set up, and the ovarian tissue is either absorbed, or becomes surrounded by a large area of dense fibrous tissue, and again the formation of ciliated epithelium is prevented.

With the object of eliminating the possibility of the formation of the ciliated epithelium lining the cysts from the epithelium covering the adductor muscle, which conceivably might have been invaginated by the introduction of the transplanting needle, a series of experiments was undertaken in which the implantation was made through a hole bored in the shell, and afterwards closed with sealing wax in the manner previously described. In these experiments the ciliated epithelial lining of the cysts developed in exactly the same way as when the implantation was made laterally through the muscle..

Another possible explanation of the development of the ciliated epithelium would be to consider that it was derived from the ciliated cells of the oviduct which ramifies through the ovary, though it would be difficult to understand how these cells could migrate to the walls of the cyst, and there form a continuous lining, while all the other parts of the ovarian tissue degenerate. To test this view two series of experiments were made. In one series only portions of the ovary taken from the extreme apex on the convex side were implanted: in this part of the ovary there is no oviduct, only the alveoli with the contained ova being present; the area thus free from the ciliated oviduct is small, but there is sufficient to make at least three implantations from each ovary with certainty that no ciliated cells are being introduced. In the parallel series of experiments portions of the ovary containing as much of the oviduct as possible were implanted. In both cases similar cysts lined with ciliated epithelium were produced. Other experiments were made in which pieces of the oviduct, which in its main branches is easily seen from the surface, were dissected out as carefully as possible, shaken in sterile sea water to remove any adhering ova, and then implanted: in these cases complete absorption and replacement by fibrous tissue often occurred, but in cases where cyst formation took place there was no formation of ciliated epithelium. Thus it is proved that the formation of this layer is independent of the presence of the ciliated cells of the oviduct.

Another point investigated was the relation of the ripeness of the ovary to the formation of the ciliated epithelium. It was found that this reaction did not take place as a result of the implantation of the spent or immature ovary, but only occurred when an ovary which was obviously full of ova, and of a bright orange or vermilion color, was used for the experiment. After the animals had been kept in the experimental tanks of the Laboratory for some time, the ovary often lost its bright color, and though full of ova became somewhat pale and unhealthy looking: experiments in which portions of the ovaries of such animals were implanted gave very uncertain results; sometimes the cysts were lined with ciliated epithelium, but more often this was absent,

and the cyst wall consisted only of fibrous tissue. If a thoroughly ripe and healthy looking ovary be used, and sepsis does not occur, it can be said with certainty that all surviving over thirty days will develop ciliated epithelium lining the cysts.

SUMMARY OF RESULTS OF EXPERIMENTAL WORK

The implantation of small pieces of the ripe ovary of Pecten maximus or Pecten opercularis into the adductor muscle of another animal of the same species results at first in the formation of a closed cyst within the muscle, lined with layers of fibroblasts. Complete degeneration and disintegration of the ovarian tissue within the cyst occurs in a few days, and then the cyst contains only an orange colored granular substance, presumably derived from the yolk, and numbers of blood corpuscles. After the lapse of from 21 to 32 days, changes occur in the innermost layer of fibroblasts lining the cyst, they revert to an embryonic type, and afterwards become converted into columnar ciliated epithelium, which forms a continuous layer lining the cyst. The changes resulting in this formation of ciliated epithelium from fibroblasts can be followed clearly step by step, and once formed, the ciliated cells persist unaltered for at least 120 days, which was the longest period for which the animals could be kept alive in the experimental tanks of the Laboratory.

Experiments were performed showing that this change is not produced by the implantation of any of the other tissues of Pecten, by neutral foreign bodies which would merely act as a source of mechanical irritation, by the transplantation of the ripe ovarian tissue of other Lamellibranchs, or by the transplantation of pieces of the ovary of Pecten opercularis into the adductor muscle of Pecten maximus and *vice versa*.

Other experiments showed that the development of ciliated epithelium does not occur if pieces of the immature or spent ovary be implanted, and that it is prevented by treating the ripe ovary with a suspension of the sperm in sterile sea water before implantation. Also that it does not occur if the ovary be killed by physical or chemical agents before implantation. A series of

experiments were made to eliminate the possibility of the origin of the ciliated epithelium lining the cysts from the ciliated cells of the oviduct, which might be present in pieces of the ovary that were implanted, or from the layer of epithelial cells forming the outer coating of the adductor muscle, which might be carried inwards by the transplanting needle.

It thus appears that the conversion of fibroblasts into ciliated epithelium is a specific reaction following the implantation of the ripe living ovary.

These observations are the result of nearly a thousand experiments, of which the majority have been performed on Pecten opercularis.

DISCUSSION OF RESULTS

It appears that the conversion into ciliated epithelium of the inner layer of fibroblasts lining a cyst formed round a piece of the ovary, which has been implanted into the adductor muscle of Pecten, is a specific reaction that occurs only when the ripe living ovary of an animal of the same species is implanted. The reaction takes place long after all trace of organised structure in the implanted tissue has disappeared, and it is difficult to conceive of its being due to any other cause than the presence of some definite chemical substance within the cyst, which is characteristic of, and specific for, each species. It cannot well be a fluid existing preformed in the ova, as in this case it would soon escape through the reticulate and easily permeable layer of fibroblasts first formed round the implanted mass, and for the same reason, considering the relatively great length of time necessary for the reaction to occur, it is not probable that it is a fluid formed at one definite stage in the process of degeneration. If a fluid at all, it seems on these grounds likely that this substance is slowly and continuously formed for a considerable period within the cyst. Examination of the contents of the cysts shows in all cases where the development of ciliated epithelium has occurred, that an orange granular substance, and blood corpuscles in various stages of

degeneration, are present. These orange granules resemble in appearance the orange colored yolk substance of the ripe ova, and the amount of this granular substance within the cysts seems to be independent of the length of time during which the implanted tissue has been allowed to remain in the muscle. If implantation of pieces of the ovary of approximately equal size have been made, examination of the contents of a cyst after 6 days will show as much of this substance present as in a similar cyst after 120 days, hence it appears that this substance cannot escape through the cyst wall. When it is considered that the development of the ciliated epithelial lining only occurs as a reaction to the implantation of ripe ova, containing a plentiful supply of the orange colored yolk substance, there is at least a possibility that the orange substance within the cysts bears a close relation to the yolk substance, and that the development of ciliated epithelium from the fibroblasts lining the cyst is a specific reaction to its presence.

Though based on no experimetal evidence, I would suggest as a possible explanation of the phenomena, that some substance is formed as a result of the ingestion of these orange granules by the blood corpuscles, and their subsequent degeneration within the cyst: that the granules themselves remain unchanged, and are again set free on the disintegration of the corpuscles, and that their action on the protoplasm of the corpuscles is merely catalytic. This substance, produced from the blood corpuscles, is probably a fluid, and would be slowly and continuously formed as long as blood corpuscles could pass through the walls of the cyst. The action of this substance on the fibroblasts forming the walls of the cyst is to delay their return to the spindle shape typical of the resting condition, and eventually to set up those changes in the inner layer of fibroblasts resulting in their conversation of ciliated epithelium. Once this epithelial layer is complete, the access of fresh blood corpuscles to the interior of the cyst would be hindered or prevented, and so the formation of this substance would tend to stop; at the same time the outer layers of fibroblasts would regain the resting state, and form a layer of typical fibrous tissue which would tend still further to prevent the ingress of fresh blood corpuscles.

An obvious objection to this theory is that the ciliated epithelium is not produced after the implantation of pieces of ovary that have been killed in any of the ways described, but it must be taken into consideration that in these cases the implantation of the dead ovarian tissue was always accompanied by a more or less intense inflammatory reaction, and so the experiments are scarcely comparable to those in which portions of the living ovary were used. Also it is probable that the chemical composition of the ovary was altered by the methods of killing.

If by future experiments, and more extended observations, it can be fully substantiated that similar cytological changes take place in other types, as a reaction to the presence of some definite chemical substance, the fact should have an important bearing on the chemical theories of development, and possibly on the origin of certain abnormal heterotopic growths.

ABSTRACT OF EXPERIMENTS PERFORMED

Total number of implantations, including animals that died before examination (about) 950
Number of animals examined in studying stages up to 20 days.............. 215
Number of animals in which cysts lined with ciliated epithelium were produced (not including those which died between 20 and 130 days, of which it is probable that most produced such cysts)........................... 68
Experiments on effect of heat on ovary before implantation 42
Experiments on effect of cold on ovary before implantation................. 18
Experiments on effect of chemicals on ovary before implantation............. 48
Experiments on effect of degeneration on ovary before implantation.......... 22
Experiments on implantation of centrifugalised ova........................ 10
Experiments on implantation of ova in gelatin.............................. 10
Experiments on implantation of foreign bodies............................. 33
Experiments on implantation of ovaries of other animals.................... 46
Experiments on implantation of ovary of P. maximus to P. opercularis........ 12
Experiments on implantation of ovary of P. opercularis into P. maximus...... 12
Experiments on implantation of male gonad................................ 20
Experiments on implantation of ovary first treated with sperm.............. 14
Experiments on implantation of portions of ovary containing no oviduct..... 12
Experiments on implantation of portions of ovary containing oviduct........ 14
Experiments on implantation of unripe ovary............................... 20

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PLATE 1

EXPLANATION OF FIGURES

1 Stages in division of a normal fibroblast. × 1000.

2 Portion of a cyst wall after 5 days. Below is the degenerating ovarian tissue invaded by phagocytes, and this is divided from the muscle by several layers of fibroblasts, mostly in the active state. Above is a blood space bounded by fibrous tissue, some of the fibroblasts of which are dividing. × 800.

3 Portion of a cyst wall after 20 days. The ovarian tissue has completely degenerated. The inner fibroblasts have changed the character of their nuclei and become connected by a continuous layer of cytoplasm. × 800.

REFERENCE LETTERS

b.c. Blood corpuscles.
b.sin. Blood sinus.
cil.ep. Ciliated epithelium.
deg.ov. Degenerating ovarian tissue.

div.fbl. Dividing fibroblasts.
fbl. Normal resting fibroblasts.
mig.fbl. Migrating fibroblasts.
msl.fbr. Muscle fibres.

msl.nuc. Muscle nuclei.

(N.B. The structure of the muscle tissue is merely indicated in the figures; details of striation, etc., are omitted.)

1

msl. fbr.
div. fbl.
b. sin.

msl. nuc.
b. c.

mig. fbl.
fbl.

fbl. lyr.
b. c.
deg. ov.

2

msl. fbr.
fbl.
fbl. lyr.
deg. ov.
b. c.

3
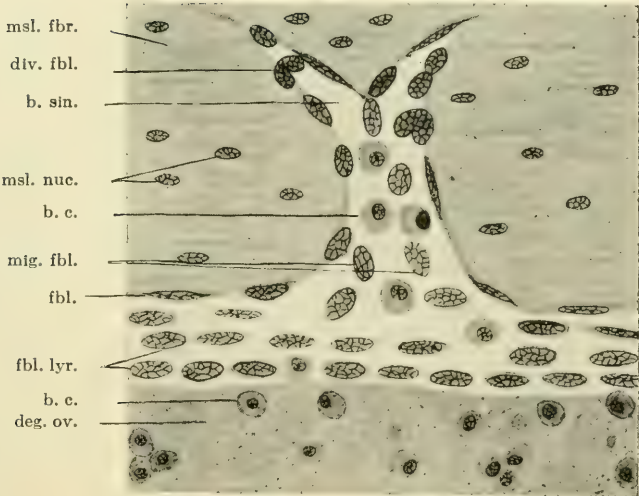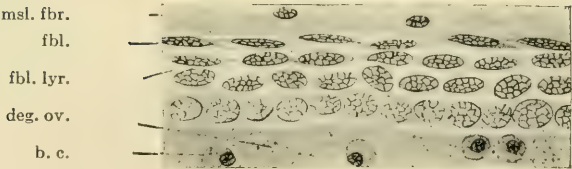
G. H. D. del.

4   Portion of a cyst wall after 23 days.   The nuclei of the changed inner layer of fibroblasts have developed definite nucleoli, the cytoplasm is more definite than in fig. 3, and the cells are suggestive of an embryonic type. $\times$ 800.

5   Portion of a cyst wall after 26 days.   The layer of cells bounding the degenerated ovarian tissue has become well defined, with a distinct basement membrane: the character of the nuclei has again altered, they are smaller and the nucleoli have disappeared. $\times$ 800.

6   Portion of a cyst wall after 30 days, showing development of long rather irregular cilia.   The fibroblasts of the outer layers of the cyst are assuming the resting stage. $\times$ 800.

7   Portion of a cyst wall after 98 days.   The formation of ciliated epithelium is complete and dividing walls have appeared between the cells.   The nuclei are smaller than in the stage represented in fig. 6. and the cilia are somewhat shorter and more regular. $\times$ 800.
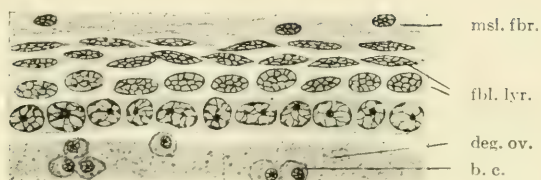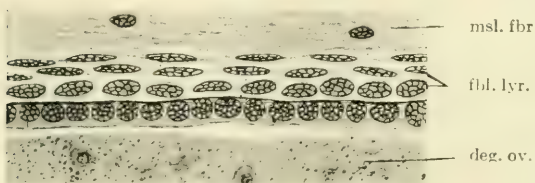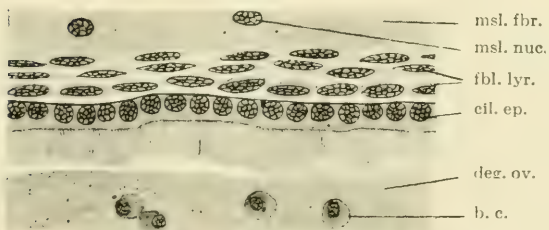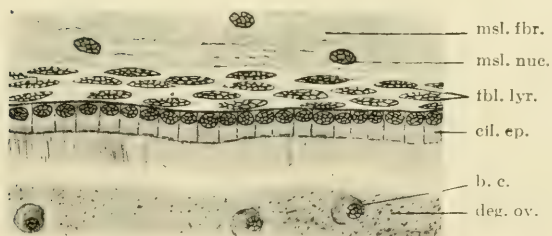
G. H. D . del.

8   Complete cyst lined with ciliated epithelium, of which fig. 7 represents a portion.   The bands of fibrous tissue on each side of the cyst are formed in the track of the transplanting needle. $\times$ 400.

b. c.   mel. nuc.   deg. ov.   fbl.   fbl. lyr.   b. sin.

msl. fbr.   b. c.   cll. ep.   fbl.

3

G. H. D. de
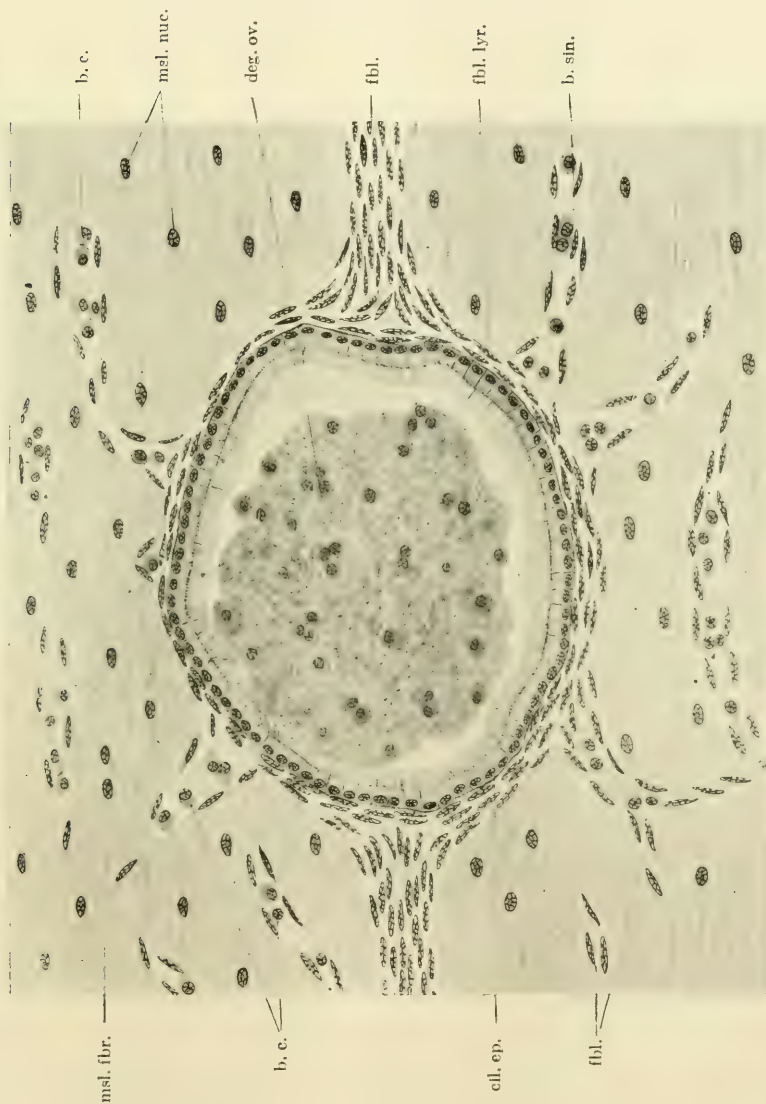
THE EFFECTS OF SEMI-SPAYING AND OF SEMI-CASTRATION ON THE SEX RATIO OF THE ALBINO RAT)MUS NORVEGICUS ALBINUS.)

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The widely accepted view of a hundred years ago that the sex of an individual depends entirely upon which of the ovaries supplied the egg is generally credited to Hippocrates (460-377 B. C.). In spite of a considerable amount of adverse evidence, this theory was revived by von Seligson in 1895, and very recently it has been advocated by Dawson ('09) and by Calhoun ('10). The first two of these recent advocates of the theory are physicians, and much of the evidence that they offer in support of their views is derived from clinical cases that have come under their own observation. Calhoun's conclusions are the results of an investigation of stock breeding on a western ranch.

Medical literature contains descriptions of many cases of one-sided ovariotomy which show that eggs capable of developing into individuals of either sex are produced in each ovary. Authentic records indicate that in man, as well as in cattle, the removal of one testicle does not lead to the production of offspring of one sex only. Evidence of this kind, however, is either ignored entirely by von Seligson, Dawson and Calhoun, or its authenticity is questioned.

Among the first to make an experimental investigation of the cause of sex in mammals was Henke (1786). This investigator operated upon pigs, dogs and rabbits, removing an ovary or a testicle from each of the individuals used in the experiments. The results reported as having been obtained when these animals

were mated are very remarkable. In every instance a litter was composed of males when the left ovary and the left testicle of the parents were lacking, and entirely of females when the operation had removed the gonad from the right side of each parent. In one of his experiments Henke mated a bitch that had been sprayed on the right side with a dog that had been castrated on the left side, but no litter was produced. From the results said to have been obtained in these experiments Henke concludes that in all mammals each ovary and each testicle has its own kind of 'germ.' Eggs from the left ovary can only be fertilized with 'samen' from the left testicle, the resultant individual always being a female; conversely, male eggs from the right ovary can only be fertilized with 'samen' from the right testicle. Most modern zoölogists would not consider these conclusions warranted, since Henke made but a small number of experiments and apparently had no controls of any kind.

Ignoring the manner in which Henke carried out his experiments, von Seligson ('95a, '95b) uses the results to support his own theory, which is that of Henke expressed in more modern terms. Von Seligson himself operated upon four female rabbits, removing the left ovary from two of them and the right ovary from the remaining two: each of these rabbits was subsequently mated twice with normal males. Von Seligson states that the two females that were spayed on the left side produced males only, and that the other two rabbits had litters containing only females. The results of these experiments can hardly be considered to afford conclusive evidence in support of von Seligson's claims, since all details are wanting regarding the manner in which the experiments were conducted. Von Seligson does not mention what precautions, if any, were taken to safeguard the experiments; and in no case, apparently, did he make an autopsy to ascertain whether or not the operation had been successful.

The publication of von Seligson's theory caused a considerable amount of discussion among physicians, particularly in Germany, and a number of papers soon appeared in various medical journals giving birth records, after one-sided ovariotomy, which were not explicable according to the theory of Henke and von Selig-

son.   Many of the writers of these papers stated their belief in
the theory that sex is determined in the ovary, but very few of
them put any faith whatever in von Seligson's contention that
male eggs are segregated in the right ovary and that female eggs
are produced only in the left ovary.

Goenner ('96) repeated von Seligson's experiments on rabbits,
and also extended them.   He removed one testicle from each of
four males and one ovary from each of five females, all of the ani-
mals being about six months old when the operation was per-
formed.   From the various matings in which animals were paired
that lacked the gonad on the same side of the body Goenner
obtained only three litters, each of which contained both males
and females.   Four of the 16˙ young contained in these litters
died before their sex was ascertained; of the remaining 12 animals,
four were females and eight were males.   These results indicate
that von Seligson did not properly distinguish the sexes of the
individuals in the various litters that he examined; but they do
not give convincing evidence against his theory, since unfortun-
ately Goenner does not state whether he killed the females and
ascertained if the gonads had been entirely removed by the oper-
ation.

Dawson's revival of the right and left ovary hypothesis for man
has one important modification: the spermatozoan is not con-
sidered to have any influence whatever in determining sex.
According to Dawson's theory, therefore, spermatozoa from
either testicle are able to fertilize eggs from either ovary.   Cal-
houn is of the opinion that the spermatozoan may possibly have
something to do with sex, and she suggests that this matter be
investigated experimentally.   Evidently this writer is unac-
quainted with much of the literature dealing with the question
of sex-determination in the higher forms.

In order to test the truth of Dawson's hypothesis, Doncaster
and Marshall ('10) made a small series of experiments last spring
on the albino rat.   One female was spayed on the right side, and
a second one on the left.   As soon as these rats had recovered
from the effects of the operation, they were mated with normal
males.   The female that was spayed on the right side gave birth

to a litter containing seven young. The sex of only five of these individuals was ascertained; four of them were females, and one a male. The female lacking the left ovary produced a litter of five young, of which three were males and two were females. The breeding females were killed soon after the birth of their litters and dissected. Each was found to lack the ovary and part of the fallopian tube on the side of the body that had been operated upon. These experiments are not open to criticism on account of the manner in which they were carried out, and the results show very conclusively that, in the albino rat, eggs capable of developing into individuals of either sex can come from either ovary. Doncaster and Marshall rightly argue that because Dawson's theory is not valid for the rat is not a proof that it is also invalid for man; but they believe, as do probably most investigators, that "definite proof for another mammal detracts from its probability." Various cases in which one-sided ovariotomy in woman has been necessitated by disease have furnished evidence against the theories of von Seligson, of Dawson and of Calhoun that is fully as convincing as is that which the experiments of Doncaster and Marshall give for the albino rat.

A number of investigators, among whom may be mentioned Rauber ('00), Beard ('02), Schultze ('03) and Russo ('09), maintain that sex is determined in the ovary, although they do not believe that the male-producing eggs are segregated in the right ovary and that the female-producing eggs are all contained in the left ovary. This theory has a considerable amount of evidence in its favor, and if it be true, it is evident, as Schultze ('03) has stated, that "in der Ovogenese ist die Lösung der Geschlechtsbildung enthalten." No advocate of this theory has ventured a suggestion as to the relative distribution of the male-producing and of the female-producing eggs in each ovary, and there is the possibility that eggs of one kind may be produced in much greater numbers in one ovary than in the other. On the current hypothesis that spermatozoa are dimorphic and that the male determines sex, the possibility also exists that many more spermatozoa of one kind are produced in one testicle than in the other. If there is a constant difference in the relative distribution of the various

kinds of germ cells in the gonads, this difference should be shown by a distinct alteration of the normal sex ratio among the young produced by mating animals from which one of the gonads had been removed. To test this point a series of experiments on the albino rat (Mus norvegicus, albinus) was started in the fall of 1909. The results obtained in these experiments are given in the present paper.

The rats used in this series of experiments were operated upon by Dr. J. M. Stotsenberg of The Wistar Institute, to whom I am greatly indebted for this assistance. In all cases the operation was performed on the rats when they were 16–20 days old, the ovary or the testicle being removed while the animal was under the influence of ether. Full details regarding the manner in which the operations were made will be given in a forthcoming paper by Dr. Stotsenberg. About half an hour after the operation the young rats were returned to their nest, and they remained with their mother until they were one month old when they were fully able to care for themselves. The sexes were separated when the animals were two months old; and the rats were mated for the first time when they were about four months old. Each pair of breeding animals, earmarked for identification, occupied one of the standard cages used for the rat colony of The Wistar Institute. It was not possible, therefore, for the experiments to be invalidated by promiscuous breeding.

The sex of a newborn rat cannot be ascertained with any degree of certainty unless the animal is killed and dissected. When rats are 14-16 days old, however, the sexes are easily distinguished, as Dr. Stotsenberg has discovered, since the mammae in the females are clearly visible at this time. After this period the hair covers the entire body. and it becomes very difficult to distinguish the sexes in the living young until they are several weeks old.

Cuénot ('99) ascertained the sex of 255 young albino rats belonging to 30 different litters. He found a slightly greater number of males than of females; the sex ratio being 105.64 males to 100 females. Records that I have made of the sex of 452 young albino rats, belonging to 80 litters, give a sex ratio of 107.33

males to 100 females.   Apparently, therefore, in the albino rat, as in man and various other mammals, there is normally a nearly equal proportion of the sexes among the young, although in all species there seems to be a slight excess of males.

THE EFFECTS OF SEMI-SPAYING ON THE SEX RATIO OF THE ALBINO RAT

Six females, belonging to two litters born in October, 1909, were operated upon when they were 16 days old.   From three of these females the right ovary was removed, and from the remaining three the left ovary was taken.   Two of these females, one spayed on the right side and the other spayed on the left side, never had a litter although they were paired with normal males for five months: both of these rats died of pneumonia when they were about nine months old.   When dissected each female was found to have but one ovary which appeared normal in every respect.   The only reason that can be suggested for the failure of these rats to breed is that they had been attacked by pneumonia when they were immature and therefore were never in a physical condition to bear young.   There is evidence that rats may suffer from pneumonia, in an incipient form, for a considerable length of time with no manifestations of the disease other than a loss of weight and a failure to breed; and it is only when this disease has nearly run its course that the characteristic difficulty in breathing, which indicates the formation of pus nodules in the lung tissue, becomes at all noticeable.

Table 1 gives a summary of the number of young produced by the four semi-spayed rats when they had been mated with normal males.   The letter R or L after the number given the rat indicates that the right or the left ovary had been removed.

Each litter of every female contained young of both sexes; and although there were more females than males in the total number of individuals that were produced, the excess of females was too small to be considered as significant.   The two females spayed on the right side had a total of five litters which contained 22 individuals; nine of these were males and thirteen were females.

The five litters produced by the two rats that were spayed on the left side contained 25 young, of which thirteen were males and twelve were females.   These results show that the sex ratio is not affected in the slightest degree by the removal of the right or of the left ovary from the breeding females, and that each ovary produces, in approximately equal numbers, eggs that are capable of developing into males and eggs that can develop into females.

Henke and von Seligson maintain that it is not possible for a male to be produced when the right gonads are lacking in the breeding pair, or for a female to develop when the left gonads have been removed.   They also state that it is impossible to fertilize the eggs of an ovary with spermatozoa from the testicle on the

Table 1

FEMALE NUMBER	NUMBER OF LITTERS	NUMBER OF YOUNG	AVERAGE NUMBER YOUNG PER LITTER	MALES	FEMALES
1 (R)......	3	12	4.0	4	8
2 (R)......	2	10	5.0	5	5
3 (L).......	3	16	5.3	10	6
4 (L).......	2	9	4.5	3	6
	10	47	4.7	22	25

opposite side of the body.   To test the truth of these hypotheses for the albino rat the following series of experiments was made:

1.   Female no. 2, which had been spayed on the right side, was mated with a male from which the right testicle had been removed. A litter containing five young was obtained; three of these individuals were males and two of them were females.

2.   Female no. 3, spayed on the left side, was mated with a male castrated on the left side.   There was one male and one female in the resultant litter.

3.   Female no. 1, lacking the right ovary, was mated with a male that had been castrated on the left side.   This female had a litter containing five young, of which three were males and two were females.

4.   Female no. 4, spayed on the left side, was mated with a male which lacked the right testicle.   The litter produced contained six young; two of which were males and four were females.

These results show conclusively that eggs from either ovary of the albino rat can be fertilized with spermatozoa from either testicle.   They also prove that males can be produced when the right gonads are lacking in the breeding animals and that females can develop when the left gonads of the breeding animals have been removed.

Table 2 gives a summary of the distribution of the sexes in all of the young produced by the four semi-spayed females.

Table 2

FEMALE NUMBER	NUMBER OF LITTERS	NUMBER OF YOUNG	AVERAGE NUMBER YOUNG PER LITTER	MALES	FEMALES
1 (R)......	4	17	4.1	7	10
2 (R)......	3	15	5.0	8	7
3 (L).......	4	18	4.5	11	7
4 (L).......	3	15	5.0	5	10
	14	65	4.64	31	34

The most striking fact brought out in the above table is that the litters average but 4.64 young each; a result which can doubtless be justly attributed to the removal of one of the ovaries from each of the breeding females.   It is very probable that in the rat ovulation takes place in both ovaries at the same time, and that the litter of a normal female contains young that have developed from eggs derived from each ovary.   Presumably, therefore, the removal of one ovary would cause a decrease in the size of the litter by lessening the number of eggs that might have been fertilized at the same time.

The average number of individuals in the 30 litters of albino rats examined by Cuénot was 8.03; while the 80 litters I have obtained contained an average of only 5.6 young.   My records, however, are made up in great part from litters produced in

inbreeding experiments, and it is well known that inbreeding causes a marked decrease in the number of offspring. A normal sister of two of the semi-spayed rats was mated four times to a normal male. She had a total of 24 young, of which fourteen were males and ten were females. The average number of individuals to a litter in this instance was six. One of the females operated upon by Doncaster and Marshall gave birth to a litter of seven young; but none of the semi-spayed rats used in these experiments ever had a litter containing more than six individuals. It seems probable, therefore, from the data shown in table 2, that semi-spaying causes a decrease in the average size of the litters, although it has no appreciable effect on the sex ratio, and apparently does not decrease the number of litters a female can produce.

Two of the semi-spayed females used in these investigations died from pneumonia; the other two were etherized when it became evident that they were out of condition and would not breed again. An autopsy was made in each instance, and in no case was there any ovarian tissue on the side of the body that had been operated upon. The remaining ovary appeard normal in every female, and there was no marked increase in its size to compensate for the loss of the other ovary, as Doncaster and Marshall found to be the case in each of the two rats upon which they had operated. As these investigators operated upon adult rats and killed them about two months after the operation, it seems probable that the noticeable increase in the size of the remaining ovary must have been due to some pathological condition, and not to a normal 'compensatory hypertrophy.' There is a considerable variation in the size of the ovaries of the same rat, as well as in those of different rats; and it is not improbable that the same ovary varies in size at different times. A series of careful measurements would have to be made of a number of ovaries, removed from females at different times in the month and at different seasons of the year, in order to obtain proper standards by which to measure any apparent deviation from the normal size.

THE EFFECTS OF SEMI–CASTRATION ON THE SEX RATIO OF THE ALBINO RAT

In February 1910, one testicle was removed from each of six males, belonging to two different litters. One male, castrated on the right side died before reaching maturity, so that two males castrated on the right side and three males castrated on the left side were available for the purposes of these experiments. Except in the cases already noted, these males were mated with normal females. The number of offspring produced, together with the distribution of the sexes in the various litters, are shown in table 3. In this table the letters R and L refer to a castration on the right or on the left side respectively.

Table 3

MALE NUMBER	NUMBER OF LITTERS	NUMBER OF YOUNG	AVERAGE NUMBER YOUNG PER LITTER	MALES	FEMALES
1 (R).......	4	17	4.1	10	7
2 (R).......	4	18	4.2	8	10
3 (L).......	2	14	7.0	9	5
4 (L).......	4	21	5.2	9	12
5 (L).......	3	13	4.3	6	7
	17	83	4.88	42	41

Practically equal proportions of the sexes were obtained as a result of this series of experiments. The two males that were castrated on the right side had a total of 35 offspring, of which 18 were males and 17 were females; the sexes were equally divided among the 48 offspring of the three males from which the left testicle had been removed. These results show that the sex ratio was not affected by the removal of the right or of the left testicle from the breeding males.

In these experiments, as table 3 shows, the average number of young in a litter was 4.88, which is very little higher than that obtained in the former experiments (table 2). The records for the litters produced by the semi-castrated males, as shown in

table 3, include the four litters obtained when these males were mated with semi-spayed females. If the records for these four litters are excluded, the remaining thirteen litters are found to contain a total of 65 individuals, which makes an average of five young to each litter. This is doubtless a low average for the litter of a normal albino rat; but it is very little lower than the average size of the litters produced in the stock colony of The Wistar Institute this past year. The small size of the litters obtained in these experiments was probably due to some external factor, and not to the castration of the breeding males.

The results obtained in these experiments indicate that, if there is a dimorphism of the spermatozoa which is associated with sex-determination, both female-producing and male-producing spermatozoa are developed in approximately equal numbers in each testicle of every normal male. A similar conclusion was recently drawn as the result of a series of investigations on the influence of the spermatozoan on the sex ratio of the toad, Bufo lentiginosus (King '11).

The following conclusions seem warranted by the results obtained in the series of experiments described in this paper. They are valid, at present, only for the albino rat; whether they can be extended to other mammals remains to be determined.

1. Each ovary produces eggs that are capable of developing into males and also eggs that can develop into females.

2. Each testicle contains spermatozoa that are able to fertilize the eggs from either ovary, and eggs thus fertilized develop either into males or into females.

3. The sex ratio is not altered in any way by semi-spaying or by semi-castrating the breeding animals. It follows, therefore, that:

a. If sex is determined in the ovary, female-producing and male-producing eggs are developed in approximately equal numbers in each ovary of the normal female.

b. If the male is responsible for sex, female-producing and male-producing spermatozoa are developed in approximately equal numbers in each testicle of the normal male.

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THE ORGANIZATION OF THE EGG AND THE DEVELOPMENT OF SINGLE BLASTOMERES OF PHALLUSIA MAMILLATA

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FOURTEEN FIGURES

In view of the fact that Driesch ('05) has stated that the eggs of Phallusia mamillata show none of the oöplasmic differentiations which I ('05) had observed in the eggs of other ascidians and since he has maintained ('96, '05) that the $\frac{1}{2}$ or $\frac{1}{4}$ blastomere of Phallusia may give rise to an entire gastrula and larva, contrary to the observations of Chabry ('87) and of myself ('05) on other ascidians, it has seemed to me desirable to reinvestigate the egg of Phallusia in order to determine, if possible, whether it differs from other ascidian eggs in these respects. A residence of several months at the Stazione Zoologica at Naples,[1] during the spring of 1910 enabled me to study the organization and cell lineage of the eggs of Phallusia and to repeat Driesch's experiments.

THE NORMAL EGG AND EMBRYO OF PHALLUSIA

The living egg of Phallusia mamillata is, as Driesch ('05, p. 661) has said "glashell und lässt nichts von den verschiedenen Stoffen Conklin erkennen." There are no pigments in the egg itself nor any visible granules of yolk or other materials. A green pigment is found in the 'test cells' (follicular cells) but this does not color the protoplasm of the egg. Furthermore I

have not been able to recognize specifically differentiated areas in the living egg, by the peculiar texture of the protoplasm, as was true in the case of Ciona. In the main these remarks are true also of the cleavage stages, the gastrula, and the larva of Phallusia; little differentiation of any kind can be seen in the living cells of these stages

Nevertheless it is quite evident that differentiations of the protoplasm must be present in the larva, at least, with its several organs and tissues, even though they may not be visible in the living condition; and these differentiations may be demonstrated by means of differential staining. The endoderm contains a substance, chiefly yolk, which stains very little if at all in Mayer's haemalum, followed by eosin; the notochord also stains very little; the muscles of the tail are stained red, while the nervous system stains blue. In the gastrula and cleavage stages substances which stain in a similar manner may be recognized in the cells which enter into the formation of these different organs of the larva. And even in the egg immediately before the first cleavage, substances showing these same staining qualities are definitely localized in certain regions of the egg.

Sections of eggs before the first cleavage show a crescent of non-granular, homogeneous substance around the posterior side of the egg, identical in position with the "mesodermal crescent" of Ciona and Cynthia. In well stained eggs this crescent is very sharply marked off from the adjoining oöplasm; it contains no yolk and is deeply colored by plasma stains. At this stage there is also a considerable amount of unstained, or faintly stained yolk which is peripheral in position at the time of the first cleavage; and there is an area of granular protoplasm around the nucleus or mitotic figure, which stains blue in the fluids mentioned above. Each of these three substances is divided bilaterally by the first cleavage plane (photos 1, fig. 2.)

Immediately after the first cleavage much of the yolk collects at the vegetative pole of the egg, anterior to the mesodermal crescent (photo 2, fig. 1); this is the area of the future endoderm cells. The remainder of the yolk lies at the periphery of the egg and ultimately goes into the ectoderm and chorda. In
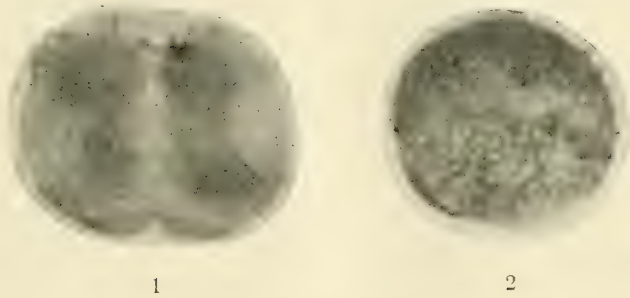
Photo 1 Equatorial section of an egg at the beginning of the second cleavage the nuclei and surrounding protoplasmic areas are in process of division, as is shown by the dark (blue) areas in the middle of each blastomere; the mesodermal crescent is shown at the posterior (lower) edge of each blastomere as a dark (red) cap; the anterior (upper) margin of the egg is rendered indistinct by the presence of test cells.

Photo 2 Section of an egg in the 2-cell stage, parallel with the first cleavage plane; with the stain used the central protoplasmic area is but faintly stained, but the mesodermal crescent, which is cut transversly, is shown as a deeply staining cap at the lower left margin of the photograph; the latter is oriented so that the posterior pole is to the left, the anterior to the right, the ventral pole above, and the dorsal pole below.

some cases, perhaps in all, the region of the egg from which the chorda and neural plate arise is stained more deeply with the haemalum than are the surrounding areas (fig. 1.)

The substances are arranged in the unsegmented egg and are later distributed to the various blastomeres in exactly the same manner as in Cynthia and Ciona. The first cleavage plane coincides with the plane of bilateral symmetry; the second divides two posterior blastomeres, containing the mesodermal crescent, from two anterior ones, containing the materials which go into the future neural plate and chorda; the third separates the vegetative halves of these four cells from the animal halves, in such manner that the mesodermal crescent and the endodermal areas are left in the vegetative half of the egg.

In all the later cleavages, the form and relative size of the blastomeres and the distribution of the various oöplasmic substances to these blastomeres are apparently precisely the same

as in Cynthia and Ciona. If eggs are fixed in Kleinenberg's picro-sulphuric mixture, and are then stained in a dilute solution of haematoxylin, in the manner described by me in former papers (1897-1905), it is possible to follow the cell-lineage of Phallusia to an advanced stage; in such study it is easy to see that the position and order of the cleavage planes, and the form and constitution of the resulting blastomeres are typically like those of other ascidians. This is true not only of the main features but also of every detail of the cleavage, such as the position and direction of the cleavage planes and pressure surfaces, and the relative quantities and positions of yolk, cytoplasm and nuclei, mesoplasm and chorda-neuroplasm in various blastomeres. In all of these details of egg organization, before and during cleavage, the eggs of Phallusia are almost precisely like those of other ascidians. On the other hand the distinction between the various oöplasmic substances, such as ectoplasm, endoplasm, meso-plasm, etc., are not so easily seen in Phallusia as in Cynthia and it is doubtful whether they would have attracted my attention if I had not been acquainted already with ascidian eggs in which these distinctions are strikingly evident; this is true of fixed and stained material as well as of the living eggs. In short Phal-lusia is not so favorably a form for the study of egg organization as is Cynthia; nevertheless eggs of Phallusia which have been care-fully fixed and stained show these oöplasmic substances in the same relative positions and proportions as do the eggs of Cynthia. The apparent homogeneity of the living eggs of Phallusia is only apparent, not real, and is principally due to the absence of pigment. Incidentally this shows, what I have emphasized elsewhere, that the pigment of the ascidian egg is not an essential or formative part of the oöplasm and that the region in which pigment becomes localized, when it is present, differs from other regions in more important respects than in the presence or absence of pigment. In other words the localization of pigment in such an egg as that of Cynthia is the result and not the cause of oöplasmic differentiation and localization.

The cup-shaped gastrulae of Phallusia, Cynthia and Ciona are composed of identically similar cells—similar in lineage,

constitution and destiny; the ectoderm, endoderm, anterior mesenchyme, caudal mesenchyme, muscle cells, chorda ring and neural ring of the gastrula are composed of wholly similar cells in all of these genera.

The elongated gastrula of Phallusia is almost identically like that of Cynthia and Ciona in the number, relative sizes and histogenetic character of its constituent cells, and in the cell lineage of its primitive organs, such as the neural and chordal plates, muscle and mesenchyme areas, ectoderm and endoderm.

The larva of Phallusia closely resembles that of Cynthia and Ciona, but all the larvae of the former hatch, whereas a varying number in the latter genera undergo metamorphosis within the egg chorion. Not infrequently slight abnormalities appear among otherwise normal larvae; the most usual form of abnormality is that in which the neural plate does not infold and the eye pigment is more or less scattered in surface cells; in other cases the notochord may show sharp bends and swellings. Such abnormal larvae frequently hatch from the egg membranes.

POTENCY OF SINGLE BLASTOMERES OF THE EGG OF PHALLUSIA

Certain blastomeres of the 2-cell, 4-cell and 8-cell stages of Phallusia were killed or injured by shaking the eggs vigorously in a vial in the manner described by Driesch (1895). In general, however, I found it easier and more effective to cause these injuries to single blastomeres by spurting the eggs from a pipette into a watch glass. The eggs were drawn into a pipette having a moderately fine point and were then spurted out with considerable force. The eggs of Phallusia are so delicate that most of the eggs treated in this way are injured in whole or in part; some of them do not develop at all, in other eggs one or more of the blastomeres may be injured so that they do not develop, while other blastomeres remain capable of development. Usually the injured blastomeres become more opaque than the uninjured ones, so that it is possible to isolate at once partial eggs in which one or more of the blastomeres have been killed.

1. *Cleavage of partial eggs.* Driesch has said that the cleavage of $\frac{1}{2}$ blastomeres of Phallusia is neither whole nor half, but 're- gellos-solid.' I also find that the cleavage of such eggs differs from the whole or half cleavage of the normal egg in that the blastomeres along the injured side turn in to a certain extent over the injured surface thus causing the living half to become spherical in form. However, the sequence, rate and differential character of the divisions remains the same as in the half of a normal egg, and the resulting blastomeres may still be identified with those of the normal cleavage.

The cleavage of right or left $\frac{2}{4}$ blastomeres is essentially like that of $\frac{1}{2}$ blastomeres, but differs in a typical manner from the cleavage of anterior or posterior $\frac{2}{4}$ blastomeres; and the latter are also different one from the other. All of the muscle-forming substance is found in the two posterior quadrants of the egg. The two small cells which mark the posterior pole in most ascidian eggs, and the rows of large muscle-forming cells which run forward from these on both sides, clearly distinguish the pos- terior $\frac{2}{4}$ embryo from the anterior $\frac{2}{4}$ one. In all of these regards the cleavage of single blastomeres of Phallusia resembles that in Cynthia. On the other hand it is undoubtedly much more diffi- cult to identify the different quadrants of the egg, and the blas- tomeres which are formed from them, in Phallusia, than in Cyn- thia, and this is especially true in the later stages of cleavage. On the whole, however, there is no reason to doubt that the form of the cleavage of single blastomeres is the same in the two genera, and that it differs from the normal, chiefly if not entirely, in the fact that in most cases the surviving blastomere becomes nearly spherical, thus causing the cells formed from it to close in over the injured side.

2. *Gastrulation of partial eggs.* There no doubt that in many cases the $\frac{1}{2}$ or even the $\frac{1}{4}$ egg of Phallusia may give rise to a stage which closely resembles a small but typical cup-shaped gastrula, but in every case which I have studied carefully this resemblance has proved to be apparent rather than real. In most cases it is due to the ingrowth of ectoderm cells over the injured side, and in these cases the apparent mouth of the gastrula

lies on this side. If this were really a typical gastrula it would
be necessary to suppose that the original polarity had been
turned so that what was at one time the transverse axis had
become the dorso-ventral axis. All the evidence available shows
that such a change of polarity never occurs. But there is also
direct evidence that these apparently typical gastrulae are not
really such. The typical gastrulae are bilaterally symmetrical,
these $\frac{1}{2}$ gastrulae are not, but invariably lack the substances and
cells of the missing half; the typical cup-shaped gastrula gives
rise to a typical elongated gastrula and this becomes a typical
larva, the $\frac{1}{2}$ gastrula does not; in some cases in which the cells
have been prevented from growing in over the injured side the
gastrula derived from $\frac{1}{2}$ of an egg may be seen clearly to be $\frac{1}{2}$ of
a typical gastrula, but there are fewer of these cases in Phallusia
than in Cynthia, owing perhaps to the fact that the protoplasm
of the egg is more labile and the blastomeres grow in over the
injured side to a greater extent in the former than in the latter.

What has been said of the cup-shaped $\frac{1}{2}$ gastrula is true also
of the elongate $\frac{1}{2}$ gastrula; so far as I have observed it is never
typical and entire, though at first sight it may appear to be so.
However a careful study of fixed and stained specimens shows
that these gastrulae are not bilaterally symmetrical but that
they lack the muscle cells and other parts of the missing half.
Usually these forms are well rounded and are covered with ecto-
derm cells on the side next the injured blastomere, but in some
cases the endoderm remains uncovered on the injured side, as
in fig. 3, and such forms are plainly $\frac{1}{2}$ of a typical gastrula.

All that has been said of the partial character of the gastrulae
derived from $\frac{1}{2}$ blastomeres, applies with still greater force to those
derived from anterior or posterior $\frac{2}{4}$ blastomeres, or from $\frac{1}{4}$ blasto-
meres. Such embryos are never entire, so far as I have observed,
and they are even less typical than are $\frac{1}{2}$ gastrulae.

3. *Larvae from partial eggs of Phallusia.* The crucial test of
the potency of single blastomeres of the egg of Phallusia is to be
found in the kind of larvae which develop from such blastomeres,
for it is possible that regulation might be incomplete in cleavage
and gastrula stages, but complete in the larva. For this reason

I have devoted especial attention to the character of the larvae derived from single blastomeres.

There are many variations of structure in such larvae, some being more defective than others. In cases where the gastrula remains uncovered by ectoderm on the injured side, the edges may fold in toward one another in later stages, until they come into contact, thus closing the open side and bending the body of the embryo, as shown in fig. 4. Here the larva is clearly a $\frac{1}{2}$ larva, although by this bending some cells at the edge of the open side are displaced more or less to the injured side. In many cases however $\frac{1}{2}$ larvae are completely surrounded by ectoderm, and they are generally more solid than typical larvae, i.e., they contain few if any cavities (figs. 5, 8). The cells of the neural plate may form a thickened mass but they never surround a neural canal. On the other hand a gastric cavity may be present, but it is usually very small (figs. 5, 8). The notochord presents a nearly typical appearance, being composed of disk-shaped cells which are disposed in a single series (fig. 4). On the other hand paired structures, such as muscle cells and mesenchyme are found only on one side, where they are frequently typical in appearance (figs. 4-6).

But while many larvae are thus plainly defective there can be no doubt that some of the $\frac{1}{2}$ larvae of Phallusia are frequently strikingly like normal ones. They may be rounded and entirely covered by ectoderm, there being no opening on the side next the injured half; they may have a well-marked body and tail; they may possess a typical notochord, an eye spot, and may have a gastric cavity surrounded on all sides by endoderm cells (figs. 9, 10). Such $\frac{1}{2}$ larvae of Phallusia are much more typical in appearance than are those of Cynthia, and it is not surprising that Driesch, who studied them only in the living condition, regarded them as typical and entire, barring a few minor defects. Among such evident defects may be mentioned the fact that the neural plate never infolds in these $\frac{1}{2}$ larvae, and the eye pigment is more or less scattered in surface cells (figs. 5 and 11), conditions which sometimes occur among entire and otherwise normal larvae (p. 397). Even in the study of serial sections it is

sometimes very difficult to determine whether such $\frac{1}{2}$ larvae are entire in the region of the "head" (cf. fig. 10); as just said the gastric wall and body wall have completely closed on the injured side and it is only by studying some typically paired structure such as the mesenchyme areas in which the atrial cavities later form, that the incomplete character of the larva can be seen. In the region of the tail, however, sections show at once whether the larva is entire or not, for here three rows of large muscle cells lie on each side of the notochord in typical larvae (fig. 7). On the other hand in $\frac{1}{2}$ larvae three rows of muscle cells are found on one side only of the notochord (figs. 6b, 8, 10, 12), and in not a single instance have I found any evidence that muscle cells occur on the other side. In a few instances four muscle cells may be seen in section of the tail, as in fig. 10, but such cases are almost invariably due to obliquity of the section. That these $\frac{1}{2}$ larvae are really not entire is most convincingly shown in cases, such as are figured in 6b and 8, where the nerve chord of the mid-dorsal line and the caudal endoderm of the mid ventral line come into contact on the injured side, while the muscle cells lie entirely on one side of the notochord. To quote the words of a former paper (1906, p. 743),"These $\frac{1}{2}$ larvae are exactly such as would result if a fully formed larva were cut in two along the median plane and the cut edges of each half then came together, the dorsal and ventral mid lines joining." The parts peculiar to the missing half are not restored, although the larvae may appear, superficially, entire.

So far as my experience goes neither anterior nor posterior $\frac{2}{4}$ blastomeres ever give rise to forms even superficially resembling typical larvae. In these forms there is no distinction of body and tail, no typical notochord, gastric cavity, neural tube, nor muscle rows. On the other hand the chorda cells, neural plate and eye spots are found only in the anterior half, the muscle cells only in the posterior half and these cells do not have their typical arrangement in rows. Each half produces only that histological type of cell which it would have produced in a typical larva, and there is no evidence that missing parts or tissues are ever restored

in the embryos of Phallusia, in spite of the fact that regulation is in general more complete than in Cynthia.

It follows from what has gone before that $\frac{1}{4}$ larvae are even more incomplete than $\frac{1}{2}$ or $\frac{2}{4}$ ones. The $\frac{1}{4}$ blastomeres never give rise to tissues or organs which they would not have formed as parts of a typical larva; an anterior $\frac{1}{4}$ produces a portion of the neural plate, eye spot and various chorda cells, but no muscle cells (fig. 11); a posterior $\frac{1}{4}$ gives rise to muscle cells but no neural plate or chorda cells (fig. 12). Furthermore no $\frac{1}{4}$ larva has the external form of a typical larva with 'body' and tail, but is only a rounded mass of cells (figs. 11, 12).

CONCLUSION

The egg of Phallusia mamillata, although it appears perfectly homogeneous and as clear as glass in the living condition, shows when fixed and stained, the same fundamental differentiations of the oöplasm as have been found in the egg of other ascidians. Just as in the cases of Ciona and Cynthia, a cap of protoplasm which stains deeply with eosin gathers at the vegetative pole immediately after the entrance of the spermatozoon into the egg, and subsequently forms a crescent around the posterior half of the egg. This crescent ultimately gives rise to the muscle and mesenchyme cells of the tail of the larva, and is thus homologous with the mesodermal crescent of Cynthia. The substances which go into the ectoderm and endoderm cells of Phallusia are histologically distinguishable and are localized in the 2-cell stage exactly as they are in Cynthia and Ciona. The distribution of these oöplasmic substances to the cleavage cells, the form of the cleavage and the cell-lineage of the principal organs of the larva is apparently precisely the same in Phallusia, Cynthia and Ciona. In the organization and early development of the egg there is no fundamental difference between Phallusia and other ascidians.

A study of the development of $\frac{1}{2}$, $\frac{2}{4}$ and $\frac{1}{4}$ blastomeres of Phallusia mamillata shows that in this species, as in Ascidia aspersa, Cynthia partita, Ciona intestinalis and Molgula manhattensis, entire larvae do not develop from single blastomeres of

the egg. In Phallusia the $\frac{1}{2}$ larvae which develop from one of the first two blastomeres may appear superficially complete, but sections show that they always lack the organs characteristic of the missing half. Anterior or posterior $\frac{2}{4}$ larvae, and all $\frac{1}{4}$ larvae are extremely atypical and incomplete. The organs which typically arise along the median plane and from both right and left halves may be present in the larvae derived from either half (e. g. chorda, enteron, neural plate), but in no case does an organ or tissue arise from cells which typically would have given rise to a different kind of organ or tissue. Thus cells containing the substance of the mesodermal crescent do not give rise to any thing other than muscle or mesenchyme, and in this sense the substances of the egg of Phallusia, which are already localized at the time of the first and second cleavages, are 'organ forming.' In these respects Phallusia is not exceptional among ascidians. There is good reason to believe that ascidians as a class are characterized by the possession of eggs having a relatively high degree of differentiation and a low degree of regulation.

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EXPLANATION OF FIGURES

The drawings were all made from sections of eggs and embryos of Phallusia mamillata, with the single exception of fig. 9, which is a drawing of a whole mount; all drawings were made with the aid of the camera lucida and as reproduced represent a magnification of about 300 diameters.   The mesodermal crescent and the parts to which it gives rise are shaded by lines; in figs. 1 and 2 the yolk is represented by spherules and the protoplasm by stipples, in the remaining figures the stippled areas represent the ectoderm, the cells of the neural plate being more closely stippled than the general ectoderm; the yolk in the endoderm cells is merely indicated by a crenated line around the nuclei, which line is lacking in the chorda cells.

1   Antero-posterior section through one of the blastomeres of the 2-cell stage, taken parallel to the first cleavage plane, showing the mesodermal crescent the central protoplasmic area surrounding the nucleus, and the peripheral yolk; the latter is chiefly aggregated in an area anterior to the mesodermal crescent, which ultimately gives rise to the endoderm; on the anterior margin of the egg, below the equator, is a deeply staining crescentic area which gives rise to the neural plate and chorda.

2   Antero-posterior section, at right angles to the first cleavage plane of an egg during the second cleavage.   The mesodermal crescent, yolk and central protoplasm are shown as in the preceding figure.

3   Oblique antero-posterior section through an elongated $\frac{1}{2}$ gastrula.   The ectoderm has not overgrown the injured side.   Only a portion of the muscle cells of one side are shown, those of the other side are entirely lacking.

4   Horizontal longitudinal section of $\frac{1}{2}$ larva showing the manner in which the ectoderm sometimes closes by the infolding of the injured side.   The notochord is typical in form but only half the normal size, and the muscle cells are found on one side only of the notochord.

5   Horizontal longitudinal section of $\frac{1}{2}$ larva, taken near the dorsal side, showing gastric cavity completely surrounded by endoderm, a row of muscle cells of the left side, the dorsal ends of a few chorda cells (unshaded), and a solid mass of neural plate cells, containing a large spot of eye pigment.

6   Oblique cross section taken near the base of the tail of a $\frac{1}{2}$ larva.   Neural plate cells (stippled) lie at the upper side of the figure, a caudal endoderm cell near the lower side and between the two is a double row of chorda cells (unshaded); muscle cells are found on the right side only.

6b   Cross section of the tail of a $\frac{1}{2}$ larva, with a large chorda cell in the center, and with three muscle cells on its right, and two neural plate cells and a caudal endoderm cell on its left; the neural plate cells, marking the dorsal mid-line, and the caudal endoderm cell, marking the ventral mid-line, have come into contact on the injured side, and there is no trace of muscle cells between them.
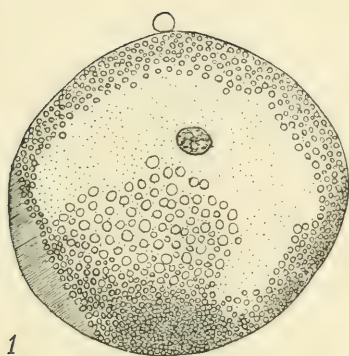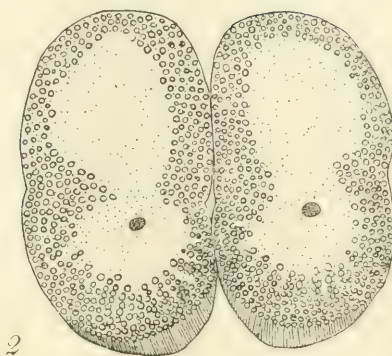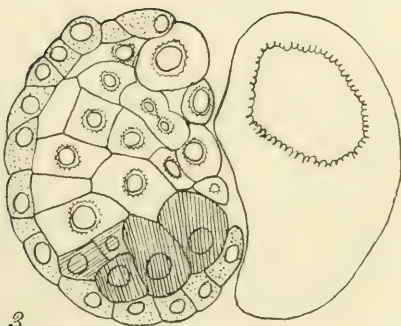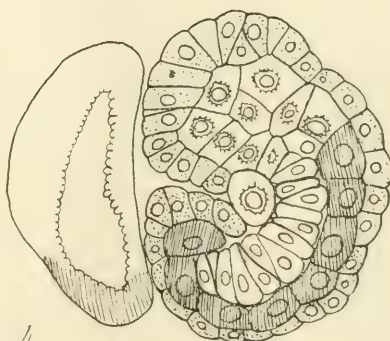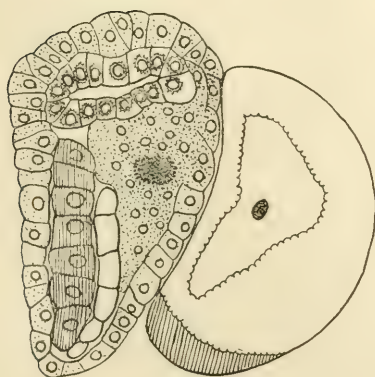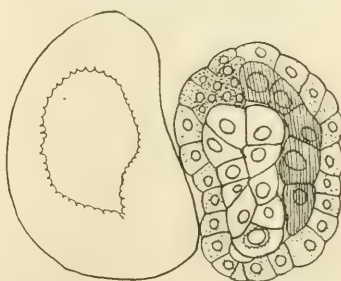
7   Cross section through the body and tail of an entire typical larva showing the nerve tube, gastric cavity, mesenchyme, chorda, caudal endoderm and muscle cells, and general ectoderm.

8   Cross section through the body and tail of a $\frac{1}{2}$ larva.   The neural plate is not infolded and the endoderm cells are not entirely covered by ectoderm cells on the injured side; the cross section of the tail is similar to that of fig. 6b.

9   Entire preparation of a $\frac{1}{2}$ larva, showing the superficial resemblance to a typical larva.

10   Cross section through the body and tail of a lateral $\frac{2}{4}$ larva in a plane similar to that of fig. 7.   The gastric cavity is nearly typical, neural plate cells are not distinguishable in the body or tail, muscle cells are on one side only of the chorda.   This is one of the most typical $\frac{1}{2}$ or $\frac{2}{4}$ larvae ever seen.

11   Section of anterior $\frac{1}{4}$ larva, showing neural plate cells with eye pigment, ectoderm and endoderm, but no mesoderm.

12   Section of anterior and of posterior $\frac{1}{4}$ larvae.   Neural plate cells are shown only in the anterior quarter, muscle cells only in the posterior quarter.
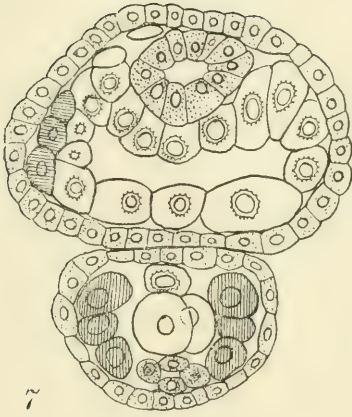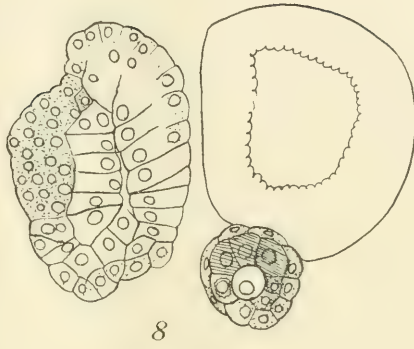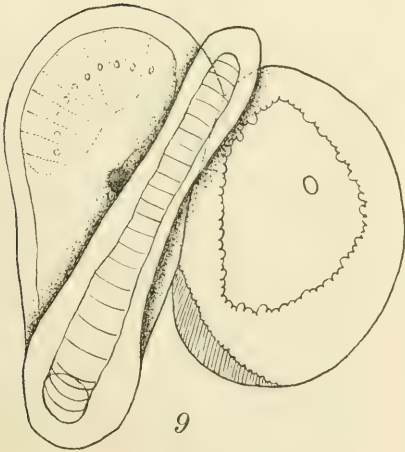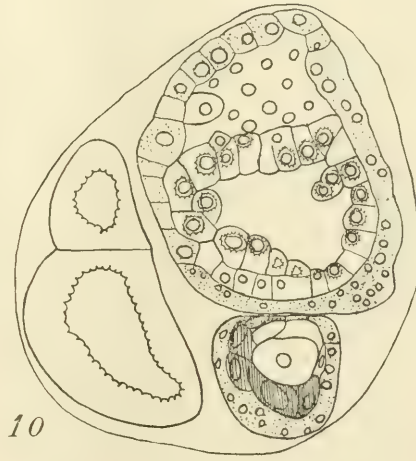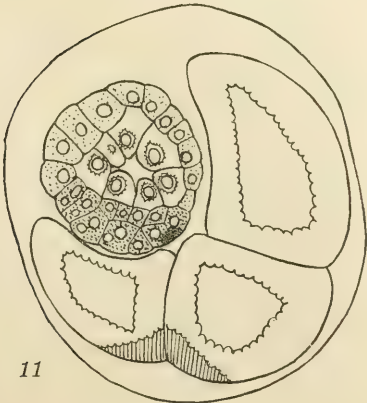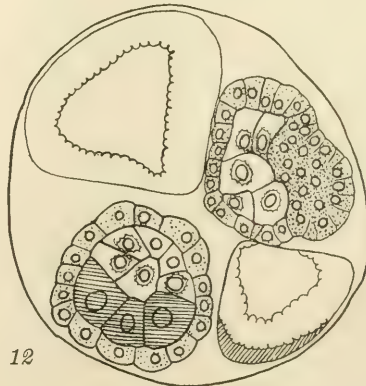
THE ADJUSTMENT OF FLATFISHES TO VARIOUS BACKGROUNDS:

A STUDY OF ADAPTIVE COLOR CHANGE[1]

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THIRTEEN PLATES

1. INTRODUCTION

That many fishes are strikingly adapted to their surroundings in respect to their general coloration is a fact familiar to all. A casual inspection of any well-stocked aquarium will reveal numerous examples. This adaptation is probably exhibited with greatest constancy by the flounders and other bottom-dwelling species, though some very striking examples are to be found among fishes which inhabit marine algae. In the latter class, a noteworthy instance is the 'sargassum fish,' Pterophryne histrio (Linn.), which dwells among the fronds of the 'gulf-weed' and with it occasionally drifts to our New England shores.

It has likewise long been known to naturalists that certain fishes possess the power of changing their colors more or less rapidly in conformity to variations in the color or the shade of the background. Those, for example, who have had much to do with the common 'minnow,' Fundulus heteroclitus (Linn.), in our laboratories at Woods Hole, realize that this fish becomes far paler when kept in a white vessel than when kept in a dark one.

The work of Pouchet[2] and a number of subsequent investi-
gators has shown that the stimuli which call forth these responses
are received through the eyes, since fishes which have been de-
prived of their sight no longer change adaptively. Blind fishes
may, it is true, undergo changes of color, but these changes bear
no relation to the optical properties of the environment. And in-
deed normal fishes may exhibit rapid changes of color, as a result
of what have been not very appropriately called 'psychic' stimuli,
e.g., fright, sexual excitement, etc. Thus Newman[3] has given us
an account of the play of colors in Fundulus majalis during 'court-
ship' and Townsend[4] has described and figured the color changes
which he has observed in fishes of a number of species in the New
York Aquarium. But changes such as these do not, so far as
we know, have any adaptive significance whatever. They are
probably of no more utility to the animal than are blushing and
various other indications of emotional disturbance in ourselves.

It now seems to be fairly certain that the immediate cause of
the color changes of fishes, as well as those of many other animals,
is a movement of the pigment granules within the chromatophores
of the skin, and not an actual contraction and expansion of the
chromatophores themsevles. The work of Pouchet, Van Rynberk[5]
and others has shown that the efferent nerve-tracts which control
this action of the color-cells pass through the sympathetic trunks.
Section of the spinal cord alone will not result in a paralysis of
the chromatophore function below this level; section of the sympa-
thetic chain will do so. But just as muscle or gland cells may be
called into activity by stimuli applied directly, without the inter-
vention of nerve fibers, so the chromatophores may undergo a

[2] The most important of this writer's contributions to the antomy and physiology
of the chromatophores are presented in the "Journal de l'Anatomie et de la Phys-
iologie," 1876, pp. 1–90 and 113–165.

[3] Biological Bulletin, April, 1907.

[4] Thirteenth Annual Report of the New York Zoölogical Society, 1909.

[5] Van Rynberk (Ergebnisse der Physiologie, Bd. 5, 1906) presents copious ab-
stracts of the work of the principal previous investigators in the field of color
change among animals; likewise what seems to be a fairly exhaustive bibliography
of the subject up to the date of his publication. For this reason, I have not thought
it necessary to cite many of these earlier papers myself.

concentration of their pigment granules as a result of mechanical stimuli, the electric current, etc. From the mechanism of color change, we should naturally expect that the chromatophores in their resting condition would be darker than when subjected to stimulus, and this appears to be the rule.

Such, in brief, are the main facts which have been recorded regarding the physiology of color change among fishes. It is my purpose in the present paper to give the results of some experiments which were conducted by me at Naples during the earlier months of the year 1910, and which were continued at Woods Hole during the succeeding summer.[6] In these studies, I have been chiefly concerned with the relations between the stimulus and the response. Very little attention has been given by me to the physiological mechanism of this response, though experiments with blinded fishes have, it is true, been performed.

While viewing specimens of one of the common European turbots, Rhombus maximus (Linn.), in the aquarium of the Naples station, I was impressed by the detailed resemblance which obtained between the markings of the skin and the appearance of the gravel on which the fish rested. Now although the pattern of the animal was such as to harmonize very strikingly with this gravel, it would not have harmonized particularly well with fine sand, even if similarly colored, nor would it have been well suited to a bottom of large stones. The query at once suggested itself: Is it a mere coincidence, this detailed agreement of the fish with its present background, or does the fish have the power of controlling the color pattern as well as the general color tone of the body?

Curiously enough, I have found scarcely any references in the literature to adaptive change in the color pattern of fishes,[7]

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[6] A report upon some of these results, illustrated by lantern slides, was presented before the research seminar of the Woods Hole laboratories last summer, and similar reports have been read before the American Fisheries Society, New York, Septe ber, 1910 (published in the 'Transactions'), and before the American Society of Zoölogists, at Ithaca, December, 1910. A brief popular statement, with five illustrations was published in the Zoölogical Society Bulletin (N. Y. Zoöl. Soc.), November, 1910.

[7] The only direct reference which I have found to differences of color pattern displayed by the same fish on bottoms of different texture, is contained in Cun-

though changes of color tone, both adaptive and non-adaptive, have been discussed by a large number of writers. That changes of the former class actually occur will be evident to anyone who devotes a moment to the inspection of my figures. Indeed they are, in many instances, so striking that it is impossible to believe that they have been wholly overlooked in the past.[8]

The fish from which I obtained the most favorable results was a small species of flounder, Rhomboidichthys podas (Delaroche)[9] belonging to the Psettinae or turbot tribe. This species occurs in various parts of the Mediterranean Sea, but unfortunately it is not so common in the Bay of Naples as one might desire for experimental purposes. Less than forty[10] specimens were at my disposal during a period of about four months; and during the latter part of my stay the supply gave out completely. For this reason, certain important tests were left untouched.

Two larger species of turbot, Rhombus maximus (Linn.) and R. laevis Rondelet, were used in a limited number of my Naples experiments; and gobies and several species of soles were likewise tried, but without any results worth recording.

At Woods Hole, my observations have been confined almost wholly to the common 'sand-dab' or 'window-pane,' Lophopsetta maculata (Mitchill), which, like Rhomboidichthys, is a representative of the turbot group. Casual observations upon the 'sum-

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ningham's "Treatise on the Common Sole," (Plymouth, '90, part iii, chapt. iii)' The author here describes and figures a spotted condition which is said to be manifested by this fish upon bottoms of gravel; but he later asserts: "All the changes are evidently due to the action of light and depend on the quantity of light acting on the sole, not on the tint or texture of the ground on which it rests."

[8] Townsend (op. cit.) mentions and figures cases in which the markings of various fishes appeared and disappeared under different conditions; and Pouchet (op. cit., p. 81–82) had earlier described a conspicuous, but apparently non-adaptive, change of color-pattern in *Callionymus lyra*. Indeed certain instances of this phenomenon are doubtless familiar to many persons, but most of such changes probably do not have any adaptive significance.

[9] This species was one of those employed by Van Rynberk (op. cit., p. 549). Strangely enough, he makes no mention (at least in the work cited, which alone is accessible to me) of the extraordinary changes of pattern displayed by this fish.

[10] Of these a considerable proportion died within the first few days of captivity. Some specimens, on the other hand, lived for many weeks, and from such my best results were obtained.

mer flounder,' Paralichthys dentatus (Mitchill), and the 'winter flounder' Pseudopleuronectes americanus (Walbaum), made it evident that these, likewise, manifested interesting pigment changes of an adaptive nature, but no systematic experiments were performed.

In the ensuing text, I shall first discuss the experiments with Rhomboidichthys, and the less searching ones upon Rhombus, at Naples; later the experiments upon Lophopsetta at Woods Hole.

2. EXPERIMENTS WITH RHOMBOIDICHTHYS PODAS.[11]

General description

When first brought into the laboratory by the collectors, the fishes were more commonly of a rather dark brown color, with inconspicuous darker and lighter markings (fig. 3c). This fact makes it seem likely that they were generally taken upon the dark, mixed sand, composed of finely divided lava and tufa, which is so common in the Bay of Naples. On the other hand, some specimens were fairly light when received, though never of a shade even approaching the maximum degree of pallor which they attained under experimental conditions. In a few instances, the intra-annular areas (see below) were white or nearly so in the freshly received specimen, giving to the latter a conspicuously mottled appearance (fig. 7). Such specimens, if we may judge from the results of my experiments, had probably come from a bottom diversified by white shell fragments.

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[11] The collectors of the Stazione Zoölogica recognize but one species of Rhomboidichthys, and Van Rynberk (op. cit.) refers to the species upon which he worked as Rhomboidichthys (mancus seu podas). On the other hand, Canestrini and others list two species, 'Rhombus podas' and 'Rhombus rhomboides,' which are said to be very similar, differing chiefly in the distance between the eyes. Moreau (Manuel d'Ichthyologie Francaise, '92, p. 469), under 'Bothus podas,' cites Steindachner to the effect that there is but a single species of which 'B. rhomboides' is the male and 'B. podas' the female. I have followed the usage of the Naples Station in referring all my specimens to the same species, although marked differences are to be noted in the shape, and especially in the distance between the eyes. Compare, for example, specimens 3 and 4, plates 6 and 7.

For an understanding of the color changes undergone by this species, some account is necessary of the permanent markings or aggregations of visible pigment. These markings are so complex in their arrangement that a satisfactory account of their condition in any one color phase would be a most laborious task, and an adequate description of their changes under various circumstances would be practically impossible. These markings may, however, be referred to several rather distinct types according to their general appearance and behavior.

1 We have a considerable number of circular, elliptical or pear-shaped spots, each bounded by a series of small white dots. The areas within these dotted outlines vary in shade, according to conditions, from a nearly pure white to a shade uniform with the ground color of the fish, however dark that color may happen to be. Some of the most striking alterations of appearance which are manifested by this fish are due to the changes undergone by this first type of spots. The rings of dots bounding these areas I shall call the 'annuli,' the areas within the latter being the 'intra-annular areas.' For the sake of brevity, I shall refer to the markings thus constituted as the 'pale spots,' where no confusion will result from this designation. So far as I have observed, the white dots making up the annuli never wholly disappear, even in the darkest condition assumed by the fish on any bottom (figs. 1c, 9d). At times, the entire areas inclosed by them are almost pure white, but the central region usually remains somewhat darker. Under certain conditions some of these spots have a rather striking resemblance to patches of lichen.

The markings of this type are arranged with a considerable degree of regularity, and it is likely that their disposition is essentially similar in all members of the species. They may be distinguished as marginal and central, according to their position. A regular alternation of annuli of different sizes (primary, secondary and tertiary) will be noted along the margin of the body. The inner edges of certain of the largest marginal annuli expand into conspicuous white crescent shaped areas. This is particularly true of one pair of primary annuli lying some distance behind the middle of the body.

Especial mention must here be made of one spot, differing in some respects from the foregoing, and lying just behind the base of the pectoral fin. This spot is elliptical in shape, and bounded by a paler outline, in which the dots are commonly not very distinct. It undergoes great and oftentimes very rapid changes of shade, and is frequently the most conspicuous white spot upon the fish. Under certain conditions of extreme contrast in the skin pattern, this spot is found to merge into adjacent white areas, so as to form an irregular white blotch of considerable size (figs. 1*j*, 1*l*, etc.).

2. Small, nearly circular spots, more regular in outline than those of the preceding type, and in some respects just the reverse of the last. Each consists of a dark ring, inclosing a center which is usually somewhat paler than itself, and the spot as a whole is usually somewhat darker than the ground-color of the fish. These spots are generally much less conspicuous than are the pale spots, and at times they practically disappear from view. At other times, however, they stand out as dark brown circles against a paler background (figs. 2*c*, 3*a*). It is probable that these spots, like those first considered, are fairly constant in their arrangement for all members of the species.

3. Large dark blotches of variable occurrence, which may better be characterized as 'permanent possibilities' than as constant features of the pigment pattern of the fish. These large blotches appear, in every case, to form around one of the spots of the preceding type (2) as a nucleus. They are conspicuous features of the skin pattern, in all cases where a blotched or highly contrasted appearance is exhibited, and it is probable that they always reappear in the same positions. On the other hand, they may be wholly wanting when the animal assumes a homogeneous or fine-grained appearance. The most nearly permanent blotches of this type are three which occur along the lateral line. The second of these, lying about two thirds of the distance from the snout to the base of the caudal fin, is the most constant of the three, and in some specimens probably never wholly disappears, even when the fish is in the most extreme con-

dition of pallor. But it is likely that dark areas may form on occasions around any of the spots of the second type.

4. As a phenomenon parallel to that last mentioned, the intra-annular areas seem at times to overflow their boundaries, thus producing larger pale blotches (figs. 1*l*, 4*d*). This fact has already been pointed out in speaking of the elliptical pale spot behind the pectoral fin.

There remain still more vague and impermanent arrangements of the pigment, which are very difficult to describe. Moreover, aside from these various types of spots and blotches, the remainder of the skin surface does not present a homogeneous coloration, but exhibits at times more or less well defined areas differing from one another in shade. Frequently, too, the outlines of the scales are conspicuously visible, contributing materially to the 'grain' of the surface.

Allowance must, of course, be made for the superficial character of the foregoing description. This crude classification of the markings of the fish is based upon external appearances only, no histological study of the skin having been undertaken. Indeed, it is probable that much of the diversity in the distribution of visible pigment in the skin of *Rhomboidichthys* is "functional" rather than "organic." Or, better stated, it may result not so much from diversity in the *distribution* of the chromatophores, as from local differences in their tonus, under the influence of the nervous system, these last being determined perhaps by the distribution of the efferent nerve fibers.[12]

Regarding the color of Rhomboidichthys, little need be said at present, since this will be discussed in relation to the various phases assumed by the fish. I need only state here that the animal is almost wholly restricted to black, white and various shades of gray, brown and yellow. It must be added, furthermore, that the browns and yellows are scarcely ever brilliant in quality, being dull tones, of a low degree of saturation. Thus they depart little, if at all, from the various hues which we encounter among the

[12] Mayerhofer (Archiv für Entwicklungsmechanik der Organismen, 1909) states that the chromatophores of the pike (*Esox lucius*) are distributed uniformly without reference to the dark crossbands of the body.

fragments of lava, tufa, shells and pottery, constituting the sea-bottom in the vicinity of Naples.[13]

In considering the appearance of this fish upon a given bottom, it must be borne in mind that the marginal fins (homologous with the dorsal and anal) are translucent, or indeed at times nearly transparent, and that through them the underlying bottom may commonly be seen distinctly. In this way, the harmony of appearance between fish and bottom is oftentimes greatly enhanced. When resting upon a bottom of sand or fine gravel, the fish frequently covers over the marginal fins, and sometimes more or less of the remaining skin surface, with the material at hand. This it does by a rapid undulatory movement of the body, by which it stirs up the sand or gravel and settles down into it.

Methods

The specimens were kept in glass jars, either circular or rectangular in form, and commonly supplied with sea-water from a large tank by means of siphons. When natural materials were employed for the background,[14] these were emptied directly into the bottom of the jar. Artificial backgrounds were produced by painting the bottom of the jar directly, or by painting glass plates which could be inserted at pleasure. An account of the different types of background employed in these experiments will be deferred until I come to a consideration of the results which were obtained.

The fishes were kept for longer or shorter periods under the various experimental conditions, notes and descriptions being made from time to time. Since verbal descriptions of such phenomena are necessarily quite inadequate, frequent photographs were made. In order that the colors assumed might be recorded with approximate accuracy, reference was in many cases made to the

[13] I do not mean by this to imply that there is any specific adaptation to this particular locality. Sands and gravels of a very similar appearance, though differing widely in their mineralogical composition, are doubtless to be found all over the world. Samples of gravel which I brought back with me may be matched, as regards their general appearance, by gravels collected in the vicinity of Woods Hole.

[14] I have throughout used the word background to designate the surface upon which the fish lay.

'Code des Couleurs' of Klincksieck and Valette (Paris, '08). Differences of color (as distinguished from shade) played, however, a minor part in my observations, partly because no very striking changes of color occurred, partly because of the difficulty of reproducing changes of this sort.

Photography.[15] The camera used[16] was mounted vertically upon a frame made for the purpose, the jar containing the fish being placed upon a horizontal platform below. With a few exceptions, the bellows of the camera was always drawn out to the same point, so that practically all of my photographs are in the same scale. This is slightly over two-thirds (69 hundredths) of the natural size. In the present reproductions, this has been reduced to approximately one-half of the natural size.

Fortunately Rhomboidichthys podas is ordinarily a quiet fish and may generally be depended upon to remain still long enough to allow of a satisfactory exposure being made. The time required for this varied, depending upon the light, from a small fraction of a second to one minute or more. The intensity of sunlight, during an average spring day in Naples, varies enormously from hour to hour, and even from minute to minute, a fact which made it very difficult in many cases for me to estimate the proper time required for the exposure. It would have been much better, had it been practicable, if an artificial light of constant intensity had been employed, and the conditions of exposure, development and printing had been identical in all cases. Thus alone would the different pictures have been strictly comparable.

As is well known by anyone at all familiar with photography, quite different appearances may be given to the same object by the use of varying technique. This is particularly true of the relative values of light and shade. An object of intermediate

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[15] I must confess to having had no experience with this sort of photography prior to the work in hand, a fact to which, I fear, some of my results bear abundant witness. My profound thanks are due to Dr. Victor Bauer, of the station staff, for much assistance and instruction.

[16] This camera was one made by Curt Bentzien, Goerlitz, and the lens by Voigtlander and Son ('Major'). Lumière and Son's 'Blue Label' plates were used, commonly 9x12 cm. in size, though in some cases 13x18 cm.

shade, lying upon a white or a black background may be caused to vary in appearance from pale gray to nearly black, without in the least affecting the value of the background. In the case of the present subject, not only might the shade of the fish as a whole be caused to vary enormously, but spots which were in reality very conspicuous could be toned down, almost to the point of disappearance. Thus it would be quite possible to produce some (though by no means all) of the differences which appear in my plates by differences of exposure, development or printing.[17] There is therefore abundant opportunity for self-deception and unintentional exaggeration of one's results, unless this source of error is guarded against. The precautions taken, in my own case, were as follows:

1. My notes upon the experiments give important clues, in respect to fishes to be compared, as for example, when one is said to be "much darker" than the other, or to have become "much paler" in the course of 24 hours. After considerable experience, one acquires fairly accurate standards of comparison and is able to pass such judgments with confidence.

2. The negative was usually developed very soon after the exposure was made, and frequently while the fish still remained upon the bottom which had been employed. Thus it was possible either to compare the negative directly with the object, or at least to pass upon the former while the appearance of the latter was fresh in memory. Likewise, prints were generally made before the lapse of many days.

3. In some cases, fishes which were to be compared were photographed together upon the same plate (e.g., some of the figures on plates 12 and 13, and many others which I have not included as illustrations).

[17] For example, as I know by actual experience, such differences as those shown in fig. 1j and 1l, plate 3, or between fig. 4d and 4e plate 7 might be brought about very readily by differences of printing; and in such a case, the backgrounds would afford little evidence of this misrepresentation. On the other hand, differences such as those between the skin patterns assumed upon gravel and upon sand or between the latter and the highly contrasted effects assumed upon some of the checker patterns would be utterly impossibe to bring about by any differences of photographic manipulation.

4.   Certain of the bottoms, notably the natural sands and gravels, form very good standards of comparison in judging of two negatives. If, for example, the familiar dark mixed sand of my experiments has the same value in two cases (either in negatives or prints) it is likely that the relative shades of the fishes have been preserved sufficiently well. Samples of these sands and gravels were saved by me and were referred to during the preparation of this paper.

5.   While making some of my exposures, I included a strip of white opaque glass, painted so as to be divided into three bands, white, black and gray respectively. This was of considerable vaule in certain cases, but its application was rather limited.

6.   Finally, a plate commonly bears in itself evidence of its having been over or under-exposed or developed, and this may be allowed for in printing.

In spite of such errors as may have crept in, owing to imperfect technique in photography, I cannot believe that the value of my results has been very materially impaired. Furthermore, I believe that the consequence of such defects of technique has, on the whole, been rather to decrease than to exaggerate the differences of pigmentation which are portrayed in my illustrations, and to minimize many of these instances of adaptation. For the color (as distinguished from the shade) was frequently an important element in the adaptation and this, of course, is not indicated in the figures. Again, the condition of extreme pallor assumed by many of the specimens upon a white background is impossible to reproduce by ordinary photographic processes,[18] since the skin of the fish always retains a certain amount of yellow, and this, as is well known, looks disproportionately dark in a photograph. Thus figures 1k, 10a, etc., give a very imperfect idea of the degree of blanching which these fishes had undergone upon a white bottom, and fig. 4i greatly minimizes the extent of the adaptation of this specimen to its gray background. According to my notes the appearance of the two harmonized very well at the time, but the photograph represents that of the former as

[18] A color screen might have been used, but this was not done.

much darker. Notwithstanding the justifiability of such a procedure in some cases, I have not taken the liberty of altering either the plates or the prints by pen or brush, not even (with a few unimportant exceptions) for the purpose of correcting imperfections or blemishes. I have, however, 'intensified' a number of negatives which had been underexposed.

In order to supplement these photographic illustrations, and to give an idea of the actual color of this species in two different phases, I reproduce (plate 6) two water-color sketches by Mr. V. Serino, one of the artists of the Naples station.[19] The artist has done well with a very difficult subject, but the constant slight changes in the disposition of the pigment of this fish proved to be extremely baffling. Moreover, the specimen chosen for these sketches, of which I have given photographic reproductions in figs. 3a and 3b, did not prove to possess the power of color adaptation in as high a degree as did many others. Thus the difference between the 'sand' and the 'gravel' phase is not nearly as striking as that shown by some other specimens. (Cf. figures 2c and 2f, plate 5).

Responses to the various backgrounds

To commence with the natural backgrounds, we have:

1. A rather coarse, mixed sand, consisting of particles of lava, tufa, shells and other materials. The general tone of this sand was very dark, since it consisted largely of jet black particles (probably magnetite, in part), but it contained abundant white and pale gray specks, which contrasted strongly with the rest. This material is of frequent occurrence in the bay, in the vicinity of Naples. Probably the majority of the fishes, as already stated, harmonized fairly well with this sand, when first brought into the laboratory. When kept upon this material, the harmony oftentimes became quite striking (figs. 2f, 4c, 9a). The resemblance consisted not only in the general similarity of color-tone, but in the granulated appearance of the fish's durface, and the presence of the white specks of the 'annuli' (see p. 414), which matched

[19] One of the many courtesies for which I have to thank the director of the station was the placing at my disposal of the services of Mr. Serino for a number of days.

the minute white particles (shell and tufa) of the sand. There was, nevertheless, nothing which could be regarded as very specific in this resemblance. It was a harmony of the same sort that we meet with frequently, both among land and water animals.

2. A fine gravel of a predominantly gray tone, apparently composed of about the same materials as the last, but much coarser, and containing a larger proportion of the paler ingredients. For the latter reason it was, on the whole, of a considerably lighter shade. The particles ranged in size from a few millimeters to about a centimeter in diameter, and in shade from pale gray or white to black. The general appearance was thus diversified, and this effect was heightentd by the presence of yellow and red fragments. Upon gravel of this type the adaptation of the fish was often very striking (figs. 1b, 2e, 5). Here is it plain that no single color or shade, disposed homogeneously throughout the animal's surface, would have rendered it inconspicuous to any such extent as this. It was necessary that the diversity of the background should be matched by a corresponding diversity on the part of the fish. The way in which this was brought to pass is difficult to analyze in detail, but the results are indicated in the figures. It is evident that the annuli have come conspicuously into view, the intra-annular areas being pale, but commonly broken up more or less by darker shading. The brown spots of the second type (p. 415) have also become rather conspicuous, and the general ground color of the skin, which had earlier been dark and fairly uniform, is now diversified by various sized patches of contrasting shades. One of the striking features of the present skin pattern is the curious appearance of transparency which is often manifested, as if we were actually looking through the body of the fish and saw the gravel underneath.[20] This effect is heightened, in some cases, by the appearance of certain of the dark spots, which are darkest at the center, and shade off into a paler margin. These spots convey the impression of depressions among the pebbles which seem to surround them. This appearance may be merely one of those

[20] This, of course, is true of the marginal fins, but is not at all true of the body proper. Moreover, even the marginal fins undergo pigment changes which contribute to the general harmony of appearance (Fig. 2e).

'accidents' of nature, of which we hear so much, but it is hard to suppress the belief that it is an integral part of the process of adaptation which we are considering.

3. A coarse gravel, composed of stones ranging from 2 to 3 or 4 cm. in diameter. This is not strictly to be included among the natural types of background, since much mud and sand had to be sifted away in order to obtain it. The stones ranged in shade from nearly white to nearly black, and the diversity was increased by the occurrence in small numbers of reddish or brown pieces. In most of my experiments, white shells and smooth fragments of marble were added in order to heighten the contrasts. This type of bottom was used with only four specimens, but rather impressive results were obtained from two or three of these. In the most striking of these (fig. 1e) most of the annuli became inconspicuous, as well as the finer elements of the skin pattern generally, and certain large dark areas came into view, giving to the fish a coarsely marbled appearance. This effect rapidly disappeared, in large degree, when the fish was transferred to a background of white glass, but reappeared upon its being returned to the coarse gravel. A similar appearance was manifested by this specimen upon certain of the other backgrounds (see below), and the same large dark areas may be distinguished in a number of the figures.[21] But the 'marbled' effect came into view clearly only in cases in which it was appropriate, i. e., upon a field having a coarsely blotched appearance.

Another specimen, which had been kept for 11 days upon the coarse gravel became in time—though rather gradually—quite strikingly adapted. Unfortunately the fish escaped from the jar and died, before any photographs were taken, but my notes state that: "for past few days mottled effect had become quite striking, showing a fusion both of darker and lighter areas into larger spots."[22] One of the other specimens, on the contrary, assumed an appearance which though plainly a mottled and 'gravelly' one, was more nearly adapted to the fine gravel than to the coarse. As

[21] This specimen seemed predisposed to the production of these effects, since the coarse blotching was dimly evident when the fish was first received.

[22] This is probably not a very accurate account of the origin of these larger spots.

regards the fourth of these specimens (no. 2), it will be seen by a comparison of figs. 2c and 2e that the appearance upon the coarse gravel was somewhat different from that upon the fine, and that the difference, so far as it went, seemed to be in the direction of a greater adaptation to the former. The case is not, however, a particularly striking one, since we should regard the appearance of the fish in fig. 2c as tolerably well adapted to the finer material. It is interesting that the coarsely blotched effect is here produced in a somewhat different manner from that shown in fig. 1e, since, in the present case, the pale spots are clearly in view all over the surface, and form a conspicuous part of the skin pattern.

Certain other materials were employed as backgrounds, which, although 'natural,' in the sense of occurring in nature, were not found in any part of the possible habitat of the species under consideration.[23] As the most important of these, we have:

4.   A very fine, jet-black sand, composed almost wholly of crystals of magnetite. This was found in the bed of a stream in the island of Ischia, and had to be separated by means of considerable washing from the paler sand and mud with which it was mixed. Although of nearly a jet-black shade, these crystals gleam so much in certain lights that the sand may appear to the observer to be far from black (fig. 1c). My chief object in using this material was to test the question whether on a bottom devoid of white particles[24] the white specks forming the annuli would completely disappear. Three specimens were put to this test. One of these was the individual which I have designated as no. 1, this being a specimen which had yielded some of my most striking results. Fig. 1c shows the condition of the fish after a stay of four days[25]

[23] This is particularly true of the magnetite sand. On account of the high specific gravity of this substance, any other ingredients with which it was mixed,—and these were all much lighter in color—tended to separate out and come to the surface. It thus seems improbable that an uncovered bed of such material should be formed anywhere upon the sea-bottom, and could ever constitute a part of the normal environment of any fish.

[24] This could of course be likewise tested by placing the fish on a black painted bottom. But the consistency of the sand seemed better adapted to calling forth normal reactions.

[25] According to my notes, the maximum effect was attained after two days.

upon the magnetite, following seven days upon the coarse dark sand already referred to. As will be seen by comparison with fig. 1a, the fish became considerably darker than it had been while upon the preceding background. The general shade of the body became darker than at any other time during my experiments, and the intra-annular areas were little if any paler than the rest of the body. The annuli themselves, however, were still clearly visible, the minute, much contracted white specks being in conspicuous contrast to the rest of the surface. Whether or not even these specks would have finally disappeared remains problematic, though I regard it as improbable, in the case of this specimen, at least. After four days, white pebbles of about the size of a bean were scattered at considerable intervals over the black surface. I thought it likely that the pale spots of the fish would come more distinctly into view under the circumstances. This did not happen, however, at least in the course of two days— an interval which was usually more than sufficient in the case of this specimen.

Two other fishes (no. 10 and no. 11) were also tested with the magnetite sand, the former for a period of six days, the latter for twelve days. The results are shown in figs. 10d and 11e respectively. It is probable that neither of these specimens became any darker than upon the ordinary dark sand. It must be stated, however, that prior to their transfer to the magnetite sand, one of these specimens had been kept all of the time, and the other most of the time, during a period of two months, upon white or pale gray backgrounds. Owing to this cause, the pale condition had become in a certain sense fixed, as later experiments showed (p. 446).

5. A very coarse sand (perhaps better, a fine gravel) composed of white, gray and dark red particles. The latter gave the material much more color than was possessed by any of the preceding materials. This sand was taken from a beach, and was not, therefore, a submarine deposit. It was employed in only a few cases. One of these was my fish no. 1, which was, as stated, a particularly adaptable one. This specimen adjusted its skin pattern very well to the texture and the general shade of the material (fig. 1d), but the reddish color of the sand exerted little or no effect upon the animal,

even during a stay of eight days upon this material. Another specimen (fig. 6c) assumed much of the color as well as the texture of the sand. This fish which, my notes state, had exhibited very little red in its composition when kept on other backgrounds assumed, after six days on the sand, a general tone resembling the no. 122 of Klincksieck and Valette. Fig. 6c does scant justice to the extent of the harmony. A third fish (no. 4) was kept upon this sand for a period of six days. Although, in general, this specimen had been a highly adaptable one, it proved to be entirely refractory upon this material, changing little, if any, from its previous condition, which was quite ill-adapted to the present type of bottom.

A very coarse gravel, likewise containing considerable red in its composition, was employed, but only one or two specimens were tested upon this, and the results were negative.

6. A white sand-like preparation obtained by grinding up fragments of marble. Upon this, certain specimens became very much paler, though none of the three fishes tested attained the same degree of pallor as those which were kept upon the marble bottom of a large aquarium or upon white glass plates. Indeed, one of the three did not undergo any striking change, even after 11 days.

7. In another experiment, large, rounded fragments of nearly black lava were used in combination with this white artificial sand, giving a vividly contrasted effect. The result, in the case of specimen no. 1 (the only one tried) was the appearance of large dark blotches and a coarsely mottled effect, resembling that shown in figure 1e or figure 1f.

8. A background just the reverse of the last was obtained by the use of rounded fragments of white marble imbedded in the coarse dark sand (1) or in the magnetite sand. The only experiment in which the latter combination was employed gave negative results, as has already been stated. The marble fragments and coarse dark sand were used with specimen no. 6, and the effect is shown in figure 6b. A considerable measure of contrast was brought about in the skin of the fish, but on the whole less than I had expected. The pale spots were at no time even approximately white.

The remaining backgrounds to be considered consisted of hard, smooth surfaces, differing thus from all of the preceding ones, which consisted of more or less finely divided materials. Thus we have:

9. The white marble bottom of a large aquarium, and strips of white opaque glass, used in the bottom of smaller jars. Under the influence of such backgrounds, all normal specimens became considerably paler in the course of a few days at the most. A number of specimens which were kept in the large aquarium for a period of 14 of 15 days became extremely pale, some of them matching very well the marble bottom, which at the end of this period was considerably discolored by a deposit of diatoms or other microscopic plants. A pure white, or even a very close approach to this, was not, however, assumed by any of my specimens, even upon bottoms of opaque white glass.[26] The closest approximation to this was a very pale yellow or straw color, not very different from the '128 D' of the 'Code des Couleurs.'[27]

10. Nearly black bottoms, made of slate or glass, painted with asphalt varnish. Upon such backgrounds several of the fishes assumed an appearance which was as dark or darker than that displayed on the dark mixed sand. In no case, however, was there in such cases a very close approach to black, while one of the fishes, although apparently normal, remained of a pale brown hue for several days.

11. A tank, much larger than the rectangular jars used in most of these experiments, having glass walls and a glass bottom, through which light was reflected from below by a mirror, inclined at an angle of about 45°. The lateral walls of the tank were covered with black cloth, and the top by a sheet of galvanized iron, painted black within. In looking into this tank from above, the observer of course perceived a well-lighted field, practically coextensive with the entire bottom. Upon first thought, it

[26] No. 11 was kept for thirty-three days uninterruptedly upon marble and white glass.

[27] This comparison was made in the case of one specimen (No. 12), which, however, probably did not become quite as pale as no. 11 and possibly some others.

would seem that the fishes should behave in such a tank much the
same as upon an ordinary white bottom of opaque material illu-
minated from above. As a matter of fact, however, only one[28] out
of seven specimens which were used actually became paler, even
after a stay of several (in one case 10) days in this tank. All the other
specimens either become darker, if they had previously been pale,
or remained dark, if taken from a dark bottom. An examination
of the actual conditions of illumination showed the reason for
this apparent anomaly. In order to see an illuminated surface
below, it was necessary that one should look directly downward,
i.e., in a direction nearly perpendicular to the glass. The fish,
however, necessarily viewed the bottom at a very obtuse angle,
and perceived, not the illuminated surface of the mirror below, but
a surface of total reflection, where the glass bottom met the air
This surface itself acted as a mirror, reflecting the darkened
walls of the tank, rising above it. What the fish actually saw,
therefore, was a dark surface, not a bright one. This could be
verified by the observer himself by viewing the bottom at a proper
angle.[29]

12. A few tests were made with glass strips painted with rather
bright shades of yellow and red.[30] The two fishes used (no. 12
and no. 4) were ones which had, under some other conditions,
shown a high degree of adaptability. One of these was kept for
five days upon the red and an equal length of time upon the yellow,
without any approach to the color of the background. The other
was kept for 10 days upon the red, with equally negative results,

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[28] This one later showed various other anomalies in its reactions.

[29] This same optical principle was responsible for another anomaly in my results
which at first baffled me. I had, in the beginning, prepared my black and white
patterns with paper and cardboard, mounted between two strips of glass, which
included, of course, a film of air. Viewed from above, the pattern was perfectly
clear and distinct, as seen through the water; but beyond a certain angle, one saw
nothing but a shining mirror. I was surprized by the complete failure of the fishes
to adjust themselves to these patterns, until I chanced to view one of the latter,
at an appropriate angle, through the side of the jar. For further references to this
point see p. 478, below.

[30] The red was of a shade closely approaching the no. 61 of Klincksieck and
Valette, the yellow of a shade similar to no. 226.

as regards color. It became of a homogeneous tone, however, having previously shown a pattern with considerable contrast.[31]

It was with various painted patterns, in black and white, that perhaps the most striking of all my results were obtained. The following backgrounds of this sort were employed:—

13. Squares of black and white, alternating as in a checkerboard. These were of four sizes, viz. 2 mm., 1 cm., 2 cm. and $4\frac{1}{2}$ cm. square, respectively. One of the most interesting of my results is the difference in the appearance assumed by the same fish upon the 2 mm. squares and the 1 cm. squares. (Compare fig. 1h and 1i; also figs. 4a and 4b). In each case, the skin presents a much more fine-grained appearance when the animal is upon the smaller squares. The effect is much as if a draughtsman had taken fig. 1h, or fig. 4b and 'stippled' the pale areas with dark ink, thus breaking them up into smaller subdivisions; at the same time doing exactly the reverse with the dark areas, by filling them in with pale dots. Thus the *degree of subdivision* of the background—independently of the relative amounts of black and white—is shown to be an important factor in the stimulus.

The 2 cm. squares, which were painted rather crudely upon the bottom of a glass vessel, called forth a somewhat interesting appearance in specimen no. 1, the only one which was used for the purpose (fig. 1f). The same large dark blotches will be seen to have come into view, as were manifested by this fish upon the coarse gravel. But the pale spots, in the present instance, are likewise conspicuous, so that there is now more contrast between the lightest and darkest portions of the surface.

The effect of the $4\frac{1}{2}$ cm. squares was tested with specimens 1 and 4. The former fish was kept upon this pattern for a period of seven days. During most of this time the animal seemed ill at ease, swimming around the jar at frequent intervals, and seldom lying upon the bottom in the attitude of complete rest. This

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[31] These experiments are confessedly too few, and their duration too brief, to permit of our forming any final opinions, even as regards this species. Other observers have recorded radical changes of color in fishes, as witness the effects of monochromatic light upon Nemachilus, as reported by Secérov (Archiv für Entwicklungsmechanik, 1909).

was in contrast to the behavior of other fishes in the room, and in noteworthy contrast to its own previous and subsequent behavior. Upon being transferred later to the 1 cm. squares, it became tranquil at once. Equally interesting was the condition of the chromatophores while the fish was kept on these largest squares. Although this was the same specimen which had shown the coarsely blotched appearance upon the 2 cm. squares, the skin now took on a nearly homogeneous appearance (fig. 1g). This was nearly or quite of the same as the appearance of this and some other specimens while swimming. Specimen no. 4 did not exhibit so complete an effacement of its previous pattern when transferred to these largest squares, but the contrasts were greatly reduced. (Fig. 4f, *not* fig. 4g, which represents the condition at the commencement of active movement). The results from the tests with this pattern are perhaps such as might have been expected on the assumption that the areas were too large to admit of their undergoing a synthesis in the creature's brain. The animal's attention has perhaps vacillated between the light and the dark areas, with a resulting indecision as to what to do.[32]

14. White (or black) circular spots, upon a background of the opposite shade. The spots were of such a diameter, and spaced in such a manner, that their aggregate area was about one fourth that of the background. These patterns were used to determine to what extent differences in the proportional amounts of black and white in the underlying surface would lead to corresponding differences in the skin of the fish. Specimens 1 and 4 were again used for this purpose, and figs. 1j, 1l, 4d, and 4e give a decisive answer to this question. The appearance of no. 4 upon the darker of these two patterns (fig. 4e) was, indeed, one of the most picturesque results which I obtained in the course of my experiments. Despite the difference between the two in the form of the spots, and the fact that the intra-annular areas were not of as pure a white as on some other occasions, the animal merged almost insensibly into its background, and was scarcely visible

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[32] The reader is at liberty to substitute less 'anthropomorphic' language, if his sensibilities are jarred by these words.

when viewed from a short distance. This seems especially remarkable when we consider that the vividly contrasted black and white condition of the fish was probably quite foreign to its experience prior to captivity.

15. Parallel alternating bands of black and white, each 1 centimeter in width. Upon these the fishes assumed an appearance (figs. 1m and 4h) not very different from that displayed on the 1-centimeter squares, i. e. the dark and light areas were highly contrasted. Nothing approaching a banded condition was manifested, and this was hardly to be expected, considering the disposition of the permanent pigment areas of the animal.[33]

16. A white plate, bearing patches of dark sand, cemented in place with Canada balsam. One fish (no. 11), after a sojourn of fifteen days on white marble, was kept upon this spotted plate for a period of eighteen days. A photograph, which was taken after thirteen days (fig. 11b) exhibits what would seem to be significant differences, in comparison with the appearance which had been manifested upon the uniformly white bottom (fig. 11a). I am not certain, however, that the somewhat speckled appearance shown in the former figure did in reality result from the character of the background; first, because these dark specks were of inconstant occurrence, coming into view on occasions and then disappearing again, and second because much the same appearance could be produced by disturbing the fish, even when the latter was on a clear white bottom. In favor of the influence of the spotted plate, on the other hand, is the fact that another specimen (no. 4) displayed a yet more conspicuously spotted surface, in the presence of scattered flakes of asphalt varnish, which had fallen upon the white bottom of the jar. The fish had previously been in the condition of extreme pallor, and devoid of prominent markings.

Upon these artificial patterns of black and white, the fishes used (most of all no. 4) lost, in large degree, the brown and yellow tints which they naturally possessed, and themselves became

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[33] Of course such a banding, even if possible, would have been of cryptic value, only if the fish lay in the same position relative to the bands, which, in fact, it did not do.

nearly black and white. Such intermediate shades as appeared were chiefly of uncolored gray. The glaringly contrasted appearance which was manifested in some cases differed very significantly from the almost complete monotone which was frequently assumed upon homogeneous backgrounds. Such natural backgrounds as the variegated sands and gravels which were used produced an intermediate effect. A more or less specific skin pattern was evoked, but this did not commonly consist of areas which contrasted very highly.

Although the capacity of the fish to assume one or another pigment pattern was obviously curtailed in large degree by a fixed arrangement of the chromatophores, and doubtless by other morphological conditions, the possibilities of reaction, within these limits, were certainly striking. Perhaps the most startling example of this diversity in spite of sameness is to be found by comparing the effects of the 1-centimeter and the 2-millimeter squares (fig. 1h, 1i, 4a, 4b). An analysis of how these various appearances were produced, from a merely descriptive point of view, would be difficult and not very profitable. Enough illustrations are here given to enable anyone to make the attempt, if he feels so inclined.

At this point, it would be well for me to qualify the impression which the reader may have received from an examination of my figures and text. It must be admitted that such changes as I have described were not of universal occurrence. In the case of some fishes, indeed, the capacity for adaptation was found to be very slight, though I do not think that it was completely wanting in a single uninjured specimen. Again, some individuals were found to undergo certain changes quite readily, while completely baffled by others. For example, specimen no. 4 'balked' most persistently when placed upon the coarse reddish sand (p. 425), and, after six days on this material, remained in much the same condition as it had been when on the banded black and white background (fig. 4h). The pale spots were distinct, and the general appearance was one of conspicuous contrasts, with little trace of red. Now this fish had throughout been one of my 'star performers,' and later showed that its capacity for change was unim-

paired. I can offer no explanation for such an utter failure to respond to a new background, on the part of a fish so well endowed with this power. Certain other specimens, as already stated, reacted adaptively to this material, one to its texture only, the other apparently to its color as well.

Another instance of the same phenomenon was offered by specimen no. 6, which, after undergoing adaptive changes on a number of bottoms (including the just mentioned red sand), finally refused to adapt itself further, and remained for thirteen days conspicuously out of harmony upon two of the artificial backgrounds.

No. 8,[34] likewise, after having adapted itself strikingly to gravel (fig. 8a), and later responding unmistakably to black and to white bottoms, remained for nineteen days in the pale condition which was induced by the last, almost regardless of successive changes to a black-bottomed jar, colored gravel, and even the familiar dark sand. Whether or not the later condition of this fish was a pathological one I cannot state, since an accident brought its life to an end. At all events, the animal could see, as was shown by its following moving objects with the eye.

Again, it frequently happened that the degree of adaptation, even after the maximum effect had been reached, varied from time to time, and sometimes became reduced to nil, even while the fish remained undisturbed. This was noticed, for example, with specimen no. 1, upon such large patterns or stones as called forth the coarsely blotched appearance. These blotches were at times scarcely distinguishable. It was thought at the time that some of these changes in the extent of the adaptedness bore a direct relation to the degree of illumination, since it was on dark cloudy days that certain of these cases of disappearing pattern were recorded. I was not, however, able to verify this conjecture.[35]

Lastly, in a few cases, after the maximum effect had been reached, the resemblance to the background seemed actually to undergo a permanent decline. This appears to have been the

[34] Cf. p. 438.

[35] Pouchet (op. cit., p. 130 et seq.) had already observed such fluctuations in the degree of adaptedness, and these he was inclined to attribute to differences in the brightness of the day.

case with no. 6, upon the background of dark sand and white pebbles (fig. 6*b*). According to my notes, the pale spots of the fish were probably more conspicuously pale two or three days after the transfer of the latter to this bottom, than they were several days later.

In spite of these admissions, it must not be supposed that my illustrations represent occasional or exceptional instances. They naturally include, among others, my best results in the way of color adaptation. But it must be stated emphatically that most of those specimens which remained in health for a long enough period exhibited these changes in a marked degree. That my most striking results were obtained from comparatively few specimens is owing to the fact that comparatively few lived for more than a few weeks in the laboratory.

Time required for these changes

This time ranged from a few seconds to several days. A change involving the almost complete withdrawal from view of the skin pigments in a dark specimen probably required the longest period. Some of the best effects which were obtained in this direction, starting with dark fishes, were observed at the close of about two weeks, when three out of five specimens which had been put in together were found to harmonize very well with the somewhat discolored marbled bottom of the large tank. The other two specimens were very pale, though still somewhat mottled. Since I was absent during the whole of this period, I do not know how soon the maximum effect appeared.

In another experiment, two specimens were recorded as being 'far from white' after nine and ten days respectively on the marble bottom, though they were 'noticeably paler' within an hour or less after being transferred to the latter. On the other hand one fish (no. 12) attained nearly or quite the extreme condition of pallor after four days in a white jar, but this had been preceded by about two weeks on various pale homogeneous bottoms.

In general, it may be said that in the experiments with natural bottoms and with certain of the patterns, the maximum effect was

commonly attained within one or two days at the most, after which no further change was manifested. The fact already pointed out by Pouchet[36] and Van Rynberk[37] that 'practice' or habituation to these changes, greatly reduces the time required, was clearly shown in my experiments with Rhomboidichthys. Certain specimens, after several changes of background, were found to adapt themselves in almost full measure to one of these within a fraction of a minute. The first of these changes had required some hours.

A case worth citing here is that of specimen 11, which had been kept for two months upon white and pale gray backgrounds. This fish, after transfer to the pure black magnetite sand, did not reach a medium shade in less than five days,[38] and continued to become darker for some days thereafter. Now this, it must be remembered, was a change toward what would ordinarily have been the normal shade. On the other hand, return to a pale gray bottom resulted in the fish becoming appreciably paler within an hour, and decidedly pale in the course of a day, although this change was away from the condition normal to ordinary specimens.

Direction of the stimulus

Before considering the question as to what part of the visual field was most effective in calling forth these transformations, a few words are necessary regarding the eyes of Rhomboidichthys. These are extremely protuberant, being virtually mounted on the ends of stalks like those of many crustacea. They are extremely motile, following moving objects in a rather uncanny fashion, or even turning without any apparent stimulus. During these movements the eye-stalks undergo an axial rotation, working together in such a manner that the two eyes nearly always look in exactly

[36] Op. cit., p. 73.

[37] Op. cit., p. 549.

[38] A. Agassiz (Bull. Mus. Comp. Zoölogy, 1892) records the production of "permanent albinos" in Gasterosteus, as a result of keeping these fishes on a white bottom. The time required for such a change is not stated, however, and no evidence is offered to prove that the alteration really was permanent.

opposite directions. The pupil of the eye is crescentic in form, owing to a semicircular projection from the dorsal side of the iris. This must largely eclipse the visual field overhead, though, as will be pointed out presently, the fish can see in this direction, even without any special movement of the eye.

In most of these experiments, glass jars were used, whose bottom areas were not large in comparison with the size of the fish.[39] Except in special cases, the walls of these jars were left unpainted. Since the fish commonly lay close to one or another side of the jar, and its head was never more than a few centimeters distant from one of the vertical walls, the animal's visual field necessarily included much that lay outside of the jar altogether. The different portions of this 'landscape' must have varied greatly in their degree of illumination, from the bright side toward the window, to the comparatively dark side viewed in the opposite direction. That the fishes actually did, at times, give attention to things seen through the walls of the jar, was evident from the fact that their eyes frequently followed my hand or other moving objects held in their vicinity. It was likewise found that the fishes could see objects directly overhead, since they sometimes rose with great celerity to take food which was dropped in from above, even in a larger tank with 25 or 30 cm. of water, and certain more timid specimens became agitated whenever I moved objects directly over them, although at a considerable distance above the surface of the water.

In the experiments thus far described, reference has been made to the bottom alone. In some cases the fish itself covered a fourth, or even more, of this bottom, while another large part of the animal's visual field was necessarily occupied by the vertical walls of the jar, together with the lights and shades of the room

[39] Most of the rectangular jars used had bottom dimensions of 15 by 20 cm. (These are the external measurements. In reality, the movable plates were somewhat smaller). Rectangular jars of a larger size were used, measuring 20 by 30 cm. at the bottom, the artificial patterns being 17×27 cm. The cylindrical jars employed had a diameter of 20 cm., and a height of 12 cm. The fishes ranged in length from 8.2 cm. to 19 cm. No. 1 had a length of 11.4 cm., no. 4 a length of 11.2 cm., no. 11 a length of 8.9 cm.

outside. Add to this the view overhead, which, as we have seen, was not wholly ignored, and it is truly a matter for surprise that the bottom was the surface chiefly concerned in evoking these pigment changes. To what degree, if any, surfaces lying in other planes might influence the fish was tested by special experiments.

A fish (no. 8) which had first been photographed on a gravel bottom (fig. 8a) was used in a series of such tests. This specimen, whose length was 12.4 cm., was placed in a glass jar having a bottom diameter of 20 cm. The bottom of the jar was painted black, but the walls were left transparent. Thus when this vessel was set into a larger jar and surrounded by gravel,[40] the latter could be clearly seen by the fish. In this contrivance, the animal became much darker in the course of the next few days, the annuli being reduced to rings of white dots. These last remained conspicuously pale, however, so that the fish was far from being concealed on this bottom. (Fig. 8b represents the condition at the close of seven days.)

The same specimen was now transferred to a contrivance exactly the reverse of the last, *i.e.*, one having the walls painted black, and the gravel visible through the bottom. A partial return to the gravel appearance was noted in the course of one day, and this increased during the next day or two, although the original appearance was not resumed in full, even after the lapse of ten days[41] (fig. 8c).

The fish was next transferred to a jar having a white bottom, 20 cm. in diameter, and black walls. Within two days it was noticeably lighter, and after three days "very much lighter and more homogeneous." It is doubtful whether any further change oc-

[40] Under water, of course, so that total reflection from the walls of the jar was avoided.

[41] The fish, after seven days, had been changed to a smaller jar, having a diameter of only 16 cm., but no certain change was noted. Gravel was now (after ten days,) placed in the bottom of the dish, so that the fish lay upon this directly, rather than upon a glass bottom. It is probable that some further change took place in the direction of adaptation to the gravel, for my notes state later that the fish now harmonized quite well. I do not believe, however, that this higher degree of adaptation had any relation to the tactile stimuli derived from direct contact with the gravel, but rather to an alteration of the visual stimuli.

curred in the next six days, at the end of which it was photo-
graphed, after being transferred to a jar having a black bottom and
white walls (fig. 8d). By this time, however, the fish appeared
to have wholly lost its power of response, for it changed but
slightly during a stay of six days in this jar, six days in a jar whose
walls and bottom were both black, five days upon a coarse reddish
gravel, and two days upon the dark, mixed sand.[42]

Similar experiments were tried with a number of specimens, but
several of them became blind or otherwise diseased before decisive
results were obtained. The most pronounced success was achieved
in the case of fish no. 9. In fig. 9a, this fish is shown lying upon the
bottom of dark mixed sand, on which it had been kept for two
days.[43] The tests to which it was further subjected were as follows.

It was first put into a dish 16 cm. in diameter and 7 cm. deep,
having the vertical walls painted black, and the bottom covered
with fine gravel, cemented in place with plaster of Paris.[44] The
length of the fish was about 15 centimeters, and it must have
covered from one-third to one-half of this bottom, while the head
must at all times have nearly or quite touched the black vertical
wall of the jar. In the course of a few hours, notwithstanding, the
fish had changed somewhat in the direction of harmony with the
gravel, while fig. 9b shows its condition on the following day. The
appearance is certainly quite different from that manifested upon
the sand, and is not ill-adapted to the new bottom, though it is
recorded as "somewhat darker in appearance than the gravel,
and gravel pattern not particularly good."

Transfer to a larger jar (20 cm. diameter, 12 cm. high), also
with black walls and gravel bottom, did not result in any appre-
ciable change, but upon removal to a large open tank, having
gravel of the same sort at the bottom, the fish resumed the maxi-
mum degree of resemblance within a few hours. The animal was

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[42] Cf. p. 433, above.

[43] It had previously been well adapted to the fine gravel. After transfer to sand,
it changed considerably within an hour or less, and the maximum effect probably
resulted within a day or less.

[44] This was done in order to prevent the animal from covering itself with the
gravel.

then returned to the larger jar used previously (20 cm. diameter, with black walls and gravel bottom), but the maximum gravel effect now persisted for two days, when the fish was again removed.

Later, this same specimen was put into another jar (20 cm. diameter) having a black bottom and white walls. After five days (no observations being made in the meantime) the fish was of a "pale brown or buff, with pale spots still lighter" (fig. 9c). My record says: "not only not black, but not a dark fish."

The animal was next transferred to a jar of the same size with bottom and walls both black. Within less than a day there was a very pronounced change: "Fish so dark and so devoid of conspicuous markings as to be pretty well concealed in jar. Not noticed at first" (fig. 9d). The transfer was again made to the white-bottomed jar, and back again to the all-black one, with similar results in each case.

From the records of the two foregoing specimens, as well as of many others, it is quite plain that the bottom, even when it is of very limited area, and largely concealed by the fish itself, may exert a predominant influence in determining the appearance assumed by the latter. On the other hand, it seems plain from the later behavior of the second of these specimens that the vertical wall may also exert an important influence upon the animal. A comparison of fig. 9c and fig. 9d will sufficiently illustrate this point for the present. This entire question was much more searchingly tested with Lophopsetta maculata (see below).

In order to determine whether a surface directly overhead would have any effect upon the color-pattern of Rhomboidichthys specimens were placed in the large tank (p. 427), lighted from below by means of a mirror. A plate of opaque white glass, of the same size as the bottom of the tank, was covered with small, irregular blotches of black paint. Four corks, 35 mm. long, were fastened to the painted surface of this plate, one near each corner, and served as legs when the contrivance was placed at the bottom of the tank. In this position, the spotted side faced downward, at a distance of about 3 cm. above the eyes of the fishes.

The three specimens used in this experiment had all been unmistakably influenced by this spotted plate when this was placed

beneath them, assuming a much blotched appearance resembling that which was commonly shown upon a bottom of fine gravel. Upon the removal of the plate from beneath them, they had returned to a nearly unspotted condition. The spotted plate, now mounted on corks, as above described, was next inserted above the fishes (under the surface of the water, of course). The plate, in its present position, was brightly lighted by the mirror below. That the fishes could see this spotted surface cannot be doubted. Nevertheless, not one of the specimens showed any appreciable influence, even after several days.[45] Return of the spotted plate to the bottom of the tank, beneath the fishes, resulted in each case in a resumption of the blotched condition within a few hours at the most.

With two specimens an attempt was made to force the animal to look directly upward. The eyes, or rather the eye-stalks, were tied together by means of threads stitched through the skin. This drastic treatment resulted in the blindness of the fishes, and no significant results were obtained.

Relation between the degree of illumination and the character of the response

The foregoing experiments were conducted in a laboratory room of medium size, lighted from one side only. Different jars were exposed to very different amounts of light, and the degree of illumination for all of them varied greatly, of course, with the weather and with the time of day. Nevertheless, no undoubted relation was discovered between the intensity of the light and the rapidity or extent of the adaptive changes. In a few instances, it is true, certain of the pigment patterns were found to be much less conspicuous on dark, cloudy days. It has been pointed out, however, that the completeness of these adaptations varied at different times in the same individual, even when no external cause was discoverable. And even if some specimens were actually affected at times by the intensity of the illumination, this was certainly not

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[45] The fishes were observed through the walls of the tank, by raising the dark curtains; also by removing the spotted plate.

the rule. The dark tone assumed by the fish upon the dark sand did not give way to a lighter shade when the animal was brought into direct sunlight; while fishes on a white bottom acquired the maximum degree of pallor, even though this white bottom was heavily shaded.

This state of affairs has already been pointed out by other observers,[46] and indeed it would seem to be a necessary one in order that the color should be adaptive or cryptic. For it is obvious that the fish itself is shaded or lighted equally with the surface on which it lies, so that the relation between the two remains unaffected by the degree of illumination.

A rather curious corollary may be drawn from this last principle. Suppose that we have two aquaria side by side, one with its inner surfaces painted white, the other painted a perfectly neutral gray. Suppose, now, that the white aquarium is heavily shaded, so as to admit comparatively little light, while the gray aquarium is well illuminated. Under these conditions, the fishes in the white tank should, according to hypothesis, assume the maximum degree of pallor, while those in the gray tank should continue to display some of their dark pigment, in amount depending upon the shade of gray employed.[47] Now experiment shows that this is precisely what happens. The fishes in the shaded white tank blanch to their fullest extent, while those in the gray tank become gray. It is obvious, however, that the dimly illuminated white bottom of the one tank may be actually *darker* than the brightly illuminated gray bottom of the other, in the sense that the former may reflect an absolutely smaller amount of light to the observer than the latter. The theoretical bearings of these facts will be discussed in a later section. At present, I shall confine myself to an account of certain experiments upon Rhomboidichthys.

Two glass jars, 20 cm. in diameter and 12 cm. deep, were used. The bottom of one of these was painted gray of a shade nearly match-

[46] Most clearly by Keeble and Gamble for schizopod and decapod crustacea (Phil. Trans. Roy. Soc., Series B, vol. 196, 1904, pp. 353 et seq.); likewise by Bauer for isopods (Centralblatt für Physiologie, 1906, p. 459).

[47] It is needless to point out that gray is not a color. If pure, it reflects white light, though of reduced intensity.

ing that produced on a color-wheel by combining two parts of black and one part of white.[48] The gray was not, it is true, perfectly neutral, being somewhat 'cold,' *i.e.*, tinged with blue. That this fact played no part in the results seems likely from my later experiments (p. 461). The side of the jar toward the window was left transparent, the opposite side being covered with white cardboard to increase, by reflection, the illumination of the bottom. In addition to this, a reflecting screen of white cloth, inclined at a suitable angle, was poised above.

The white-bottomed jar had walls which also were painted white, but the light was largely cut off from its interior by a cylinder of sheet iron, painted black, which encircled the jar and projected upward for a distance of 12 cm. above its top.

That the bottom of the gray tank was actually far lighter than that of the white tank, *i.e.*, reflected far more light, is readily seen from fig. 11*f*–12*b* (plate 13), which reproduces a photograph taken in the laboratory with the jars arranged as nearly as possible in the same manner as during the test.

Several experiments were made with these jars, with more or less instructive results. Only one of these tests, however, was so clear cut and decisive that it deserves to be described in detail. Specimen no. 11, which had previously been kept for a long period upon white and other pale backgrounds, but was in the present case taken from the black magnetite sand (after twelve days) was placed in the gray-bottomed jar. It became appreciably paler within an hour, much paler within a day, and, after two days "probably no darker than the gray bottom." Specimen no. 12 which had likewise been on various backgrounds, but came, in this instance, directly from gray, was placed in the (shaded) white jar. This specimen grew noticeably paler in the course of a day, and the pallor increased for a day or two more. (The color-book was here referred to for comparison.)

At the end of four days, the two specimens were examined under identical conditions of illumination, when it was found that no. 11 (on the gray bottom) was decidedly darker than no. 12 (on

[48] Contrary to what one might suppose, such a gray is far from being dark.

the white bottom). Photographs were now made, in which the two fishes were taken together, first on the gray bottom (fig. 11*g*-12*c*, plate 13), then on the white bottom. Both of these showed very plainly the difference of shade between the two fishes.

The specimens were then transposed, no. 11 being placed upon the white bottom, no. 12 being placed upon the gray bottom. After two days, no. 11 appeared to be paler than 12, and after four days it was certainly so. They were compared, as before, and again photographed together, first upon the gray bottom (fig. 11*h*–12*d*), then upon the white bottom. The relative shade of the two fishes had obviously been reversed.

The two animals were once more transposed, and with similar results, though no photographs were taken.

Is the behavior of the fish influenced by the degree of its adaptation to the background?

As has already been pointed out, most specimens covered themselves more or less with the gravel or sand in which they lay. In some cases, the marginal fins only were concealed; but in a few instances the entire body was buried, only the eyes protruding. So far as my observations go, the fish was just as likely to cover itself with the bottom material when its color and pattern were highly adapted to this as it was when they were glaringly ill-adapted. When specimen no. 10 was taken from a pale background and placed in black sand, it buried itself with extreme rapidity, and remained completely concealed. In this instance the fish was at the outset utterly out of harmony with the bottom. It was noted, however, that this tendency to hasty concealment beneath the sand was just as marked after the fish had assumed a shade not far different from that of the latter.

In contrast to the last example, specimen no. 11, though even more conspicuously out of harmony with its background, did not, at first, make any endeavor to bury itself when placed on the magnetite sand.

In order to test the question whether the fish, when offered the choice, tended to select a background in harmony with its own

shade, a plate of glass was employed as a bottom, having an area of 17 x 27 cm., and divided transversely into a black and a white half. Fishes nos. 11 and 12 were used in these experiments. Both were healthy and active. The former was, at the time, adapted to a very pale bottom, the latter to the dark sand. The fishes, one at a time, were placed upon this background, in the neighborhood of the division between the white and black halves, and in such a position that they could plainly see both. This experiment was repeated a number of times with each fish, the latter being left in some cases for more than an hour upon this bottom. No preference was shown for one surface more than the other. The fish commonly remained very near to where it was placed, whether or not it was adapted to the surface which immediately surrounded it, and in no instance crossed over into the opposite side. On the contrary, it happened that in more than one case, the dark fish moved further back into the white area, and vice versa.

Each of these fishes was then placed upon bottoms of dark and of white sand. No. 11 showed no disposition to burrow in either. No. 12 covered itself very little with either sand, and with one no more than with the other.

Such experiments are of course not entirely conclusive, but, when taken in connection with other observations, they at least render it improbable that the fish exercises much selection in respect to the shade of its background. The behavior of this animal is thus not at all in accord with that of the decapod crustacean *Hippolyte varians*, as described by Gamble and Keeble.[4]

The rôle of sight in these reactions

Various previous observers[50] have recorded that blind fishes failed to undergo adaptive color changes, and it has been pointed out that both specimens which have become blind through natural causes,

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[49] Quarterly Journal of Microscopical Science, 1900, p. 601. (See especially plates 32 and 33.)

[50] *E.g.* Pouchet, Mayerhofer and Secérov, in works already cited.

and those which have been deprived of their sight experimentally, cease to adjust themselves to the shade of their background.

Certain of my own fishes which failed to respond adaptively to changes of bottom were found to have become diseased in one or both eyes, although, as has been stated, some of the most refractory individuals had not lost their sight. One of the fishes, both of whose eyes were affected, acquired a peculiar appearance which was not noted in any normal specimen. It assumed a rich brown color, with specks of orange, the whole effect being much more decorative than the somber hues ordinarily displayed by this species. Another specimen, one of whose eyes had already become blind, took on almost precisely the same appearance after I had cut the optic nerve of the sound eye. In both cases the fishes remained conspicuously out of harmony with the gravel or sand on which they were kept.

My most complete experiments in blinding fishes were later made upon Lophopsetta, but a few which were made with Rhomboidichthys deserve recording. It was at first my endeavor to cover the eyes with some opaque coating, without thereby causing any injury.[51] This proving impracticable, I next tried the effect of searing the corneas with a red-hot platinum wire. The results, with specimens no. 10 and 11, are detailed in the next paragraph. The effect of cutting the optic nerve has already been described for one specimen[52]. I will add that this fish was subsequently kept for 22 days upon a white marble bottom. A slight paling was noted at first, and this, as subsequent experiments showed, might have occurred equally well on any bottom. Thereafter, no change was noted, and the fish remained fairly dark as long as kept under observation.

One result of blinding, manifested in two of the foregoing specimens, is of peculiar interest. Specimen no. 10, which had been

[51] Mixtures of lampblack with certain fatty substances were tried, but it was found that these would not adhere for more than a very few minutes.

[52] Aside from the resulting loss of sight, it is probable that there is a distinct shock effect from the cutting of the optic nerves. Thus specimen no 10 (see below), when in a pale condition after the searing of the corneas, turned dark immediately, upon the cutting of the optic nerves, and this dark condition presisted until the death of the fish four days later.

kept most of the time during a period of two months upon white and pale gray bottoms, was transferred to the black magnetite sand for a period of six days. At the end of this time the fish was nearly or quite as dark (fig. 10d) as are most of these fishes when adapted to a very dark bottom. The animal was then blinded by searing the corneas with a red-hot platinum wire. The effect was a conspicuous paling of the body (fig. 10e), which became evident in a short time and persisted for some days, after which the darker condition began to return.

Substantially the same results were obtained from specimen no. 11, which had been kept for an even larger proportion of the preceding two months upon pale bottoms, and had been only 3 days upon the black sand at the time of blinding. Within a few hours, the fish returned completely, or nearly so, to the extremely pale condition which it had acquired upon its earlier backgrounds, and remained in this condition for a day, after which the observations were brought to an end.

These results are readily intelligible on the assumption that the pale condition had, to a certain extent, become fixed in the nervous mechanism during the long sojourn of the fish upon such bottoms. The transfer to a dark bottom resulted, without much delay, in the acquirement of a darker appearance, but this condition was not, at first, a stable one, and its maintenance seemed to depend upon the continuance of visual stimuli. There are, it is true, certain facts which seem irreconcilable with this hypothesis, as we shall see later. But the foregoing experiments have been repeated upon two other species, and the facts themselves are beyond question. Moreover, fishes which had undergone no extended sojourn upon a pale bottom did not (with a single exception) become pale when blinded.

Changes having no relations to visual stimuli

As stated in an earlier paragraph, decided changes occur in the disposition of the skin pigment, which have no relation to the background or, indeed, to any visual stimulus whatever. Certain very conspicuous phenomena of this sort were observed

during the course of the present experiments. The fishes commonly assumed a very different appearance when disturbed or when swimming 'voluntarily' in the aquarium from that displayed when at rest.[53] Such changes generally followed certain laws, though there were abundant exceptions to these.

1. A fish of pale or medium shade generally became darker[54] when disturbed, and at such times dark spots or blotches commonly came into view.[55] In a few specimens highly colored red specks appeared at such times. The resting condition was resumed in a few minutes or seconds after the fish settled upon the bottom. These phenomena were manifested even by those blinded specimens which had secondarily become pale.

2. In certain cases very dark fishes, which had recently been considerably paler, assumed a lighter hue when caused to swim around. These changes were so inconspicuous that I was not at first certain of their reality, but their ocurrence was confirmed by observations upon at least three fishes, after transfer to the magnetite sand.

3. When the fish was in the highly contrasted condition, with conspicuous white and black areas, this appearance commonly diminished, or even wholly disappeared, when the animal was disturbed. Its skin then assumed a medium shade, and the markings became inconspicuous. The same monotone was commonly assumed when these fishes swam about without known external stimulus. Indeed, in the case of certain specimens, I found it a very easy matter to discern the fish's 'intention' to begin swimming by the disappearance of the spots and the assumption of this monotone. Thus fig. 4g was taken upon such an occasion. Upon settling down upon the bottom, the skin pattern gradually came into view, and generally attained its maximum distinctness within comparatively few seconds. The effect of these latter changes

[53] Pouchet, Van Rynberk, Townsend (op. cit.), and others, have called attention to pronouced color differences, in some species, between the resting condition and conditions of activity or excitement.

[54] This darkening, under the influence of disturbance, is the only change of this sort recorded by Van Rynberk, who believes it to be of constant occurrence.

[55] Pouchet (op. cit., p. 76) records the appearance of such spots in the turbot.

upon the observer was much like that which one experiences in watching the development of a photographic plate. Indeed it not infrequently happened, in those cases in which the maximum adaptive effect was not displayed by the fish at the time of its being disturbed, that this maximum effect appeared for a brief period after the animal settled down, only to diminish again after a few moments.

3. EXPERIMENTS UPON RHOMBUS

Although Rhombus maximus was the species which first arrested my attention in the show aquarium by its extraordinary adaptation to the gravel bottom, no striking results were obtained in the laboratory from the single specimen which I used. Moreover, the species was too large for convenient manipulation.

Two specimens of Rhombus laevis were, however, used with some interesting results. Both of the specimens showed a high degree of adaptation to the fine gravel, used in the foregoing experiments, and one of them (the other was not tested) likewise acquired a high degree of harmony with the dark sand. Both specimens became much paler when placed upon the white marble bottom of a large aquarium, though neither attained such an extreme condition of pallor as did Rhomboidichthys. One of the two, at the end of a stay of forty-six days, "harmonized pretty well with the now much stained marble bottom," though the maximum degree of adaptation had probably been brought about long before this. Even after this extended sojourn upon the marble, however, I note of this specimen, after transfer to gravel, that "within a short time, certainly in less than an hour, the spots had come distinctly into view, and on the same afternoon the fish harmonized pretty well with the gravel."

After a short sojourn on the gravel, the corneas of this fish were rendered opaque by the application of silver nitrate, the animal being then returned to the same bottom. After the lapse of a day, the fish was very much paler than before the operation, and not far different from the condition when on marble. After two days, however, the gravel condition (*i.e.* the darker, spotted

condition) had, to a considerable extent, reappeared. Subsequent experiments with this fish led to the suspicion that enough light still passed through the corneas to influence the changes. After complete extirpation of the eyes, the fish, which was finally returned to the marble-bottomed tank, remained "rusty brown, with inconspicuous markings."

4. LOPHOPSETTA MACULATA

Since certain important points were left in an unsatisfactory state by the experiments at Naples, this line of work was resumed during the following summer in the laboratory of the Bureau of Fisheries at Woods Hole. The fish which was chiefly employed in these later experiments was the common 'window-pane' or 'sand-dab,' Lophopsetta maculata (Mitchill), another member of the turbot group. Lophopsetta proved to be a far less favorable object for studies of this sort, since, on a white surface, it never attained such an extreme degree of pallor as did Rhomboidichthys, and its capacity for displaying adaptive skin patterns, though far from wanting, was much more restricted.[56] The experiments with this species were therefore concerned chiefly with the relative influence of different portions of the visual field, the time of reaction, effect of blinding, etc. Especial attention was likewise given to the problem of how the fish is able to conform the shade of its skin to that of its background, irrespective of the degree of illumination.

A few experiments with natural bottoms (gravel and sand) were also tried, but without any very striking results. In the large exhibition aquaria the adaptive reactions were, however, sometimes rather impressive. Some specimens assumed a characteristic appearance upon gravel, which was decidedly different from that displayed upon sand, and the changes were sometimes fairly rapid. Upon the former material, spots, both light and dark, came into view rather conspicuously, while upon the latter,

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[56] Indeed, of the nine species of Pleuronectidae and Soleidae which have been observed by the writer, Rhomboidichthys podas appears to possess by far the highest capacity for adaptive changes of this sort.

a fairly homogeneous brown or buff tone was commonly assumed. The adaptations of Lophopsetta are not to be compared, however, with such appearances as those exhibited by Rhomboidichthys. The rich mosaic effect sometimes displayed by the Mediterranean species would seem to be structurally impossible in the American one. As a compensating advantage, from the experimenter's point of view, the latter may be obtained in far greater numbers.

The experiments with this fish were nearly all performed in the cod-hatching boxes of the Woods Hole station. These are wooden boxes, with a bottom area of 30 x 70 centimeters, and containing water to a depth of 18 centimeters. They are built in rows of 12 each. In the course of the present investigations, they were painted variously, as will be described in connection with the separate experiments. No photographs of Lophopsetta were taken.

Blotched surfaces

A few experiments were tried to test the capacity of this species to adapt itself to a highly contrasted pattern. Four specimens were placed (two at a time) in one of the boxes just described, the walls and bottom of which had been painted white with small irregular daubs of black scattered throughout the entire surface. All of these fishes responded unmistakably to this stimulus, the ground-color becoming (or remaining) medium pale, while certain stellate or irregular black blotches came distinctly into view. The fishes thus acquired a piebald appearance, quite different from anything which was observed o: a background of uniform shade.

Another interesting case was noted, in which these dark blotches appeared upon an extremely pale specimen which had been kept for three days in a box having white (unspotted) walls and bottom. It was found, in this instance, that patches of very dark vegetable debris had accumulated at the bottom of the box in the neighborhood of the fish's head. Removal of this debris resulted in the disappearance of the spots, while a later accumulation led to their return. It must be remarked, however, that in the specimen under

consideration the dark blotches were vaguely visible much of the time, even when the fish was on a homogeneous ground. The presence of dark spots in its neighborhood merely served to accentuate these.

Direction of the stimulus

A point which was tested much more thoroughly than at Naples was the relative influence of the bottom and of the vertical walls of the receptacle in determining the changes of shade on the part of the fish. The bottoms employed were, as just stated, 70 centimeters long and 30 centimeters wide. The length of the fishes varied from 24 to 35 centimeters, and their area probably ranged from 200 to 400 square centimeters, or from 10 to 20 per cent of the area of the bottom. The fishes lay, much of the time, near one or another wall of the box, and the larger specimens naturally could not at any time reach a position very far removed from the latter.

Boxes were employed having 1, walls and bottom painted black; 2, walls and bottom white; 3, walls black, bottom white; 4, walls white, bottom black. In addition to this, false walls of galvanized iron were made, which were painted white or black or both. These could be inserted into any of the boxes without disturbing the fishes.

1. In boxes of the first type, the fish became (or remained) as dark as it was capable of being. The shade varied, of course, with the specimen, but was usually a very dark brown, and fairly homogeneous, though certain small white spots sometimes showed distinctly. Usually the fishes were very inconspicuous in the black boxes.

2. In the white boxes, the fishes commonly attained a considerable degree of pallor, assuming a shade which may perhaps best be characterized as buff, i.e., a pale yellowish or brownish gray. In this condition, while of course far less conspicuous than previously, they could not be regarded as very well concealed, at least from a nearby observer. The harmony with the pale bottom was furthered by the fact that, with the withdrawal from view of the dark pigment, not only the marginal fins (already partially transparent), but the adjacent portions of the body proper, be-

came fairly translucent, so that the underlying surface was more or less visible through them.

3. In the black-walled, white-bottomed box, different fishes behaved differently, depending upon the individual peculiarities of the specimens, or upon their previous treatment. When placed here in the dark condition, most specimens remained fairly dark, even after a lapse of some days. But they were, notwithstanding, commonly affected somewhat by the white bottom, being noticeably paler than those in an all-black box, and in some cases exhibiting a peculiar blotched or marbled appearance, as if attempting to adapt themselves to black and to white at the same time.[57] In one instance, a fish became nearly as pale as did the average specimen in an all-white box.

Two fishes which had been placed in this box in the dark condition, and which had remained dark for four days, were transferred to an all-white box. In the latter they attained nearly or quite the maximum degree of pallor within a single day. Upon being returned to the black-walled, white-bottomed box, they remained pale for two days, *i.e.*, as long as they were kept there.

Two other specimens, which had remained dark for two days in this box, became pale in seven hours or less when the white movable wall was inserted. Upon removal of the latter, at the end of one day, however, the fishes promptly began to darken, and became nearly or quite as dark as before. Yet another two, which were put in when pale, remained so for two days.

Thus, while there can be no doubt as to the influence either of the white bottom or of the black walls in these experiments, the relative importance of the horizontal and vertical surfaces seems to differ in different cases. The same is seen to be true of the next sort of box considered.

4. In the white-walled, black-bottomed box, dark fishes in every case remained fairly dark, though they were in some cases influenced by the white surfaces around them, for about half of

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[57] No such contrasts were here produced, however, as were shown upon the spotted bottom. (See above.)

the specimens took on a somewhat paler shade, looking as if a very thin 'wash' of white had been spread over their bodies.[53]

Pale fishes placed in this box either became nearly as dark as when kept in an all-black box (three specimens), or at least of a medium shade (one specimen). One of these specimens acquired the mottled appearance referred to above.

5. The movable walls, when painted uniformly, were used in order to change the outlook of the fish without otherwise disturbing it. The results obtained from their use were the same as those following the transfer of the animals from one tank to another. They need not detain us here. Highly interesting results were obtained, however, with a wall painted partly black and partly white, the line of division between the two areas being horizontal. In the first experiments the white and black portions were of equal extent, that is to say, the wall, which was 18 centimeters high, was divided into a white and a black half. Later, it was painted so that the white band occupied only a fourth of the height of the wall.

The movable wall, thus painted, was used only in the white-bottomed, black-walled box. It served, therefore, to add a certain amount of white to the vertical surfaces of the box. Some of the results from its use seem worth recording in detail.

Two fishes (dealt with together) which had remained dark in this box, were found to become pale when an all-white movable wall was introduced, returning to the dark condition, however, when this was removed. The half-white, half-black wall was then inserted, the white half being uppermost. No change occurred, even after 8 hours. Upon the reversal of the wall (white half now

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[53] Here, and in all similar cases, it was absolutely necessary to place fishes together in the same box before comparing them. The white walls, in the present case, reflected enough light upon the surface of the animal to give it a paler appearance than when in an all-black box. Accordingly, specimens from one of the latter were transferred to the present box, and a comparison made with the fishes which had been kept in this for some time. The reverse change was likewise made, both lots being compared together in all-black tank. A mirror was also used in examining specimens kept in dark boxes, the fishes being illuminated by reflected light. Without such precautions, one may easily be led into error in judging of the less pronounced changes of shade.

down) the fishes became decidedly pale within an hour. A second inversion of the wall resulted in the fishes becoming much darker (about medium shade) in $4\frac{1}{2}$ hours. It would seem, however, that even in this position the white surface did have some influence, since the fishes did not become very dark until the removal of the wall (leaving the vertical surfaces entirely black), when they became so within two hours.

Two other specimens were, at the outset, subjected to the same tests as the preceding ones, and with substantially the same results. After several further changes of the visual stimulus, they were again subjected to the influence of the black-walled, white-bottomed tank, in which they now displayed a somewhat intermediate shade. The black-and-white metal wall was next put in (now one-quarter white), the white band being below. After twenty minutes, one fish had acquired nearly the maximum degree of pallor, but the other had undergone no change.

Two more fishes kept in this box for two days, remained dark, one being of about maximum darkness, the other somewhat paler. The black-and-white wall (one-quarter white) was now inserted, the white band, as before, being below. After two days, both fishes had become pale, though not of maximum pallor. After removal of the wall, they remained in this condition for a day.

Thus, it is interesting to note that when the bottom and the adjacent zone upon the vertical walls were white, even though this zone were no broader than $4\frac{1}{2}$ centimeters, the fishes reacted much (though not quite) the same is in an all-white tank. When, on the other hand, the bottom was white, and the adjacent zone upon the vertical walls was black, even the presence of an overlying band of white, 9 centimeters wide, was not sufficient to call forth a truly pale condition in any of the four specimens thus tested.

It would hence seem, at first glance, that quantitative relations alone could not determine which of these two components of the visual stimulus should prove effective. In endeavoring to decide this point, however, one must distinguish between the potential and the actual visual fields. What the fish, from a given position,

can see is not necessarily the same as what it commonly *does* see. It may well be that the animal's attention is chiefly centered upon areas which do not rise much above a horizontal plane. I shall discuss this point more fully later.

Rapidity of these changes

The average time required by Lophopsetta to attain the highest degree of pallor, commencing with the dark state[59] was probably less than two days, and the change was commonly noticeable within a single day. One particularly refractory specimen was kept for four days in a white box before any undoubted change occurred. The change, when it did come, was rather abrupt, though the highest degree of pallor was not attained until the lapse of 6 to 7 days. Specimens were found, on the other hand, which changed decidedly within a few hours, when placed for the first time in a white tank, and, in one case at least, the maximum degree of pallor was assumed in less than twenty-four hours.

After the first experience of this sort, it happened, in many cases at least, that subsequent changes were undergone much more rapidly. Thus specimens which required several days for the first change to the pale shade often completed this change within a few hours, after one or more such transpositions. One dark fish, for example, when placed for the first time in an all-white box, showed little or no evidence of paling after one day, and did not blanch to the fullest extent until the lapse of about three days. After being returned to black, it was recorded, at the end of 19 hours, as being nearly or quite as dark as originally. When transferred to the white box for a second time, the fish became decidedly paler in less than a minute, and within an hour was nearly or quite as pale as at any time previously.

Whether or not a similar shortening of the reaction-time may be brought about in the case of the reverse change, *i.e.*, from light to dark, was not fully determined. A change in this direction is, in

[59] Nearly all of the specimens, when first brought into the laboratory, were much nearer the darkest than the lightest condition. They were, too, frequently kept for some days before being used, in a large stock tank, painted black within.

most cases, a return to a more nearly normal state, and presupposes a previous (commonly recent) change from an original dark condition.

Condition in total darkness

The fishes were examined at night, after three hours or more of darkness, by means of an electric flash-light. This was done on two different occasions, and with a considerable number of fishes in various conditions. With one or two possible exceptions, these fishes were of nearly or quite the same shade as when last observed in the daytime.[60] Even specimens which had but recently assumed the pale condition were found to have retained this after the withdrawal of the visual stimulus. Certain observers have reported among fishes characteristic differences of color during 'sleep' or at least at night.[61] I have found no evidence of such in the case of Lophopsetta.

Experiments were tried in which fishes of different shades were shut up in a light-proof box. In the first of these, two specimens which had become very pale and two of maximum darkness were put into the box together. After five days the two dark ones were found to be dead. The other two, though much darker than when put in, still remained distinctly paler than those kept in neighboring black boxes. They assumed the darkest condition after a few hours' exposure to light in such a box.

In the next experiment, one pale and one dark fish were kept in the light-proof box for a period of seven days. The pale specimen had previously passed 6 days in an all-white box. When the fishes were examined at the end of their stay in darkness, the dark specimen was found to be as dark as before; the other, though now fairly dark, was distinctly paler than the former. It acquired the darkest shade, however, after a few hours' exposure to light in a black box.

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[60] In making such comparisons, I could only refer to my own notes describing the condition of these fishes in the daytime, and to my recollection of this. Differences may have appeared which escaped me.

[61] E. g. Verrill (American Journal of Science, 1897, p. 135). Verrill's observations were made by dim gas-light, mainly between midnight and 2 a.m.

Thus it is plain that the shade assumed by the fish under the influence of visual stimuli tends to be retained for a considerable period after the latter are withdrawn.[62]

Experiments with blinded fishes

Any method of permanently destroying the sight of an animal must necessarily involve a considerable nervous shock, and it might be fairly objected, in the lack of further evidence on this point, that such results as are described below may be due, in part at least, to this shock, rather than to the loss of sight alone. Thus any mere failure to respond adaptively after the operation is not, in itself, a decisive proof that vision is a necessary element in the reaction. Such doubts are, to be sure, greatly weakened in the present instance by the fact that the blinding of one eye was found to have little or no effect upon most specimens.

In order to meet fully this objection that we may have to reckon here with a 'shock' effect, I endeavored in the first place to use a bandage of black cloth, fastened over the eyes. It was necessary, however, to stitch this bandage to the margin of the head, and this, of course, involved an injury to the fish. Moreover, the friction or pressure of the cloth soon damaged the eyes and led to blindness.

Accordingly, I gave up all attempt to blind the animals without inflicting injury,[63] and adopted the plan of cauterizing the surface of the eyes with silver nitrate. This resulted at once in an opacity of the cornea. After the lapse of a few days, the latter fell from the eye, exposing its interior to the surrounding water. Even in this condition, the retina (or optic nerve) frequently remained for some days (7 or 8, in certain cases) decidedly sensitive to light, as was shown by reflecting daylight upon the head with a mirror, or by the

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[62] For the pike, Mayerhofer (op. cit.) regards darkness as a "strong stimulus to an extreme contraction of the chromatophores," since fishes which were thus kept became much paler after a few days. On the other hand, Secérov and some others report the acquirement of a deeper shade in total darkness.

[63] The use of a coating of opaque material had been found to be impracticable with Rhomboidichthys.

use of a flash-light at night. In many cases the eyes moved unmistakably, or the fish even swam away, as a result of the stimulus.

Altogether, 16 specimens were deprived of the sight of both eyes by cautery, while three others were blindfolded. Of this total, 8 fishes were in the dark condition at the time of the blinding; 9 were in the pale condition, and two others in an intermediate state. As regards results, the following general statements may be made:

1. Dark specimens, excepting those having the history specified below (3), remained dark after the destruction of sight.

2. Pale specimens, after blinding, remained as pale as before for about a day, after which they gradually grew darker, and became indistinguishable from those which had been blinded when in the dark state. The duration of this peristence of the pale condition after blinding seemed to bear little relation to the length of the previous sojourn upon a pale background. Thus fishes which had been kept in white boxes for only two days before being blinded retained the pale condition about as long as specimens which had been thus kept for fourteen or seventeen days.

3. Specimens which had passed considerable periods (seventeen to twenty-five days) in a white or pale gray box, and then, before blinding, returned to black just long enough to cause them to resume the earlier dark shade (twenty-four hours, or less), became pale again within a few hours after blinding, and remained thus for about a day, after which they gradually became dark again. Three of the four specimens thus treated reverted, after blinding, to nearly or quite the maximum degree of pallor; the fourth became distinctly paler, though not so pale as it had been. The results of these experiments upon Lophopsetta are thus in complete agreement with those obtained from the use of Rhomboidichthys and Rhombus. On the other hand, with a single exception (see below), none of the ordinary dark specimens became paler as the immediate result of blinding.

4. The shade assumed by the blinded specimens was not thereafter influenced in any appreciable degree by the background.[64]

[64] One apparent exception is to be recorded among all the specimens used. This fish was of a fairly dark shade at the time of blinding. Some hours after transfer

Change from all-black to all-white boxes called forth no visible response.

5.   Whatever the original shade of the fish, that which was finally assumed was, as already stated, a dark one.   But the final condition was not, in the majority of cases, that of maximum darkness.   It was frequently a shade distinctly paler than this, though in all cases one nearer to the darkest than the palest condition. Certain blinded specimens displayed a distinctly abnormal appearance which I never observed in an uninjured fish.   On the other hand, some specimens remained very dark, and of normal appearance, to the end.   For example, one fish (pale when blinded) was of about the maximum degree of darkness, even after forty-one days.[65]

With six specimens, the sight of one eye only was destroyed. In three cases, this was the right eye, in three others the left. Since the two eyes are rather differently directed with reference to the bottom, I thought it worth while to look for a possible difference in the effect of the two operations.   Of these six specimens, four retained the power of adaptive change nearly or quite unimpaired.   Indeed one of these, for rapidity and completeness of the adjustment, remained one of the most favorable specimens which I encountered.

Of the two remaining fishes, one appeared to have very largely lost the power of change while in the other, this was considerably

to a white receptacle, the animal was found to be very pale.   It must be borne in mind, however, that the immediate result of cauterizing the eyes was not complete blindness, but that the corneas were merely rendered opaque.   In this exceptional specimen the opacity might not have been complete.

. [65] According to Pouchet, the shade assumed by a blinded turbot was always an intermediate one.   Mayerhofer, experimenting upon pike, found that the immediate effect of blinding was a paling of the fish, this being followed by the assumption of a more intensely colored condition than before the operation, accompanied by a disappearance of the dark bands.   The further history of the specimen depended upon whether it was kept in the dark or in the light.   If the former, the pigment tended to disappear.   If the latter, the pigment cells not only persisted on the back and sides, but developed upon the (normally pale) ventral surface.   This last phenomenon suggests the artificial production of pigment upon the lower side of flounders in Cunningham's well-known experiments.

impaired. In both of these cases, it was the right eye[66] which had been destroyed, but I do not regard this fact as of any significance, since in the third specimen thus treated there was little or no impairment of the pigment reactions.

Since a very decided inhibition of these reactions was also noticed in certain specimens which were injured in other ways, without being blinded (p. 465), it seems probable that the shock of injury and not the loss of the sight of one eye was responsible for such impairment of the chromatophore function as was observed in these last cases. This, of course, is reason for suspecting a similar shock effect, perhaps an even greater one, in the case of those fishes both of whose eyes had been destroyed. Certain facts which I have recorded above may indeed be referred to this cause. But it must not be forgotten that we have, quite apart from these blinding experiments, conclusive evidence of the part played by sight in these reactions.

Relation between the degree of illumination and the shade assumed

In discussing the experiments upon Rhomboidichthys, I pointed out that the degree of illumination of the background had little or no effect upon the reaction which the fish underwent. As a special illustration of this principle, it was shown that a fish became paler upon a white bottom, even though this was heavily shaded, than upon a gray bottom exposed to a considerable measure of light. The latter surface, in my experiments with Rhomboidichthys, was shown photographically to be very much lighter than the former.

Identical results were obtained with Lophopsetta. One of the boxes was painted gray of a shade close to no. 499 of Klincksieck

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[66] *I.e.*, the eye which belonged morphologically to the lower, unpigmented side of the body. Pouchet (op. cit., p. 88) had just the opposite experience, finding that one turbot whose left eye was destroyed failed to respond as well as previously, though no such impairment was observed in a specimen whose right eye was detroyed. He suggested a possible physiological correlation between the left eye and the skin of this (the upper) side.

and Valette.[67]   The gray paint used was not perfectly neutral, it is true, being, when fresh, slightly tinted with blue. After exposure to sea-water, however, it changed somewhat, becoming 'warm' (*i.e.*, slightly yellow) instead of 'cold.' It was probably in this condition during most of the period of the experiments.[68]

The gray box was situated near the window and was well lighted throughout much of the day, though not exposed to direct sunlight.

Another box was painted white within, but its interior was rather heavily shaded by a tent-shaped contrivance of galvanized iron. This was painted black within, and had a long cleft in the top, partly for the admission of light, partly to permit of observations. The cleft was largely closed by strips of wood, the amount and distribution of the light being thus controlled.

No photographs were taken of the surfaces upon which the fishes lay, but I feel sure that the difference in the amount of light reflected from the two bottoms was as great, or greater, than in the experiment with Rhomboidichthys.

Four fishes were used in the present experiment. Two specimens at once were kept in each of the two boxes. From time to time, those from one box were transferred to the other box, for brief periods, in order that all four might be compared directly under identical conditions of illumination and of background. Such direct comparisons were also made with other pale fishes kept in a neighboring white tank which was well lighted.

At the close of the first phase of the experiment, the two specimens in the gray box were found to be decidedly darker than those in the shaded white box. More pigment was visible in the skins of the former than in those of the latter, and they were of a gray appearance, in contrast to the yellowish or slightly pinkish appearance of those in the white box. The latter, moreover, were

[67] Prof. Yerkes, who kindly matched a sample of this paint upon a color wheel, reports that 75% of black and 25% of white gave the desired shade.

[68] If anyone wishes to maintain that this slight element of color (aside from shade) probably played some part in the results, I cannot absolutely refute him. I can only say that my experiments as a whole make this seem to me highly improbable.

found to show no appreciable differences of color or shade from specimens which were kept in a well lighted box, having a white interior.

The two sets of fishes, which had now been in their respective boxes for six to nine days, were next transposed, *i.e.*, those from the gray box were transferred to the covered white one, and vice-versa. At the end of 5 days, the relations in respect to pigmentation were found to be reversed, those which had formerly been darker now being paler and vice-versa. One of those which had previously been very pale was now (when on gray) recorded as "one of the best cases of resemblance in respect to general color tone which I have had."[69]

Reference should be made here to one exceptional specimen which, when dark, was placed in the shaded white box for three days with little or no effect. Upon being removed, to an *unshaded* white box, on the contrary, some change was noticeable during the same day, while on the next day the fish was very pale. In the case of this specimen, therefore, it would seem that the scant illumination of the interior of the former box had exerted an inhibitory influence.[70] On the other hand, this result may have been due merely to my having dealt with a rather refractory specimen, which was on the point of changing at the time of removal from the shaded box, and would have done so if left there.

How is the fish able to adjust itself to a bottom of given shade, independently of the degree of illumination?

As was pointed out earlier, (p. 441) it is plain that, in order that the change of shade on the part of fish should be *adaptive*, the latter would have to behave exactly as described. When, however, we begin to inquire as to the visual stimuli responsible

[69] Indeed, a number of specimens, aside from those employed in the present experiment, harmonized quite strikingly with this and other gray bottoms which were used. This harmony was enhanced by the transparency of the fins and marginal portions of the body, but was also due, in no small measure, to a disappearance of the yellow and brown tones and the assumption of a nearly pure gray.

[70] It may well be that the degree of illumination at times affects the *rate* of adjustment (Cf. Mayerhofer, op. cit., p. 554), but not its *character*.

for the changes just recorded, the case becomes decidedly puzzling. For anyone with any knowledge of optics knows that gray—at least a perfectly neutral gray—is not a color. Such a gray reflects all the components of white light in their normal proportions. It differs from white only in this, that it reflects a smaller fraction of the total quantity of light which falls upon its surface. Gray is thus relatively darker than white, but not always absolutely darker. When we ourselves judge of an object as being gray or white we make an allowance for the degree of illumination to which it is subjected, and this last is inferred from the totality of the visual field.

But how about the fish? It is not in the position of an outside observer, with abundant standards of comparison at hand. This tank, with its painted surfaces, would seem to constitute for the time being its entire environment. How, then, if the walls of the shaded white box reflect absolutely less light to the animal's eyes than do those of the brightly lighted gray box, does the creature take on a lighter shade in the former than in the latter?

So far as I can see, we are limited to two alternative explanations: either (1) the fish takes into account the degree of illumination, just as we do, and makes due allowance for this in judging of the paleness or darkness of the background; or (2) it makes a direct visual comparison between its own surface and that of the background and endeavors to bring the former into harmony with the latter.[71] In this second case, since the body of the fish itself is lighted or shaded to an equal extent with the background, it would have to become fully white in order to conform even to a dimly lighted white background.

Let us take up the latter of the foregoing alternatives first. In order to test the question whether the fish compares its own appearance with that of its background, I have tried the expedient of concealing from the view of the animal its own skin color. For

[71] Such hopelessly 'anthropomorphic' language may shock the sensibilities of the ultra-mechanistic reader. I therefore hasten to explain that no consciously reasoned mental processes are here implied. The whole chain of events could doubtless be stated in purely physiological terms, were we more familiar with the facts, but so, for that matter, might our own behavior.

this purpose, I have employed two methods, that of staining the skin of the fish and that of covering it with a cloth, stitched along the margin of the body.[72] At first I made a full-length 'swimming suit' for the animal, but this did not seem necessary, since from the position of its eyes, only the anterior portion of the body can fall within its range of vision. Accordingly, a mask only was employed in my later experiments, apertures being made for the eyes.

Now I have devoted considerable time and trouble to experiments of this sort. Eighteen fishes were provided with cloth masks or coverings for the body, and 8 others were stained in various ways.[73] Fully satisfactory tests were, however, found to be difficult, if not, indeed, impracticable. It was, for example, very hard to cover the head completely with cloth, and at the same time permit of an unobstructed view for the eyes. In the earlier experiments, the body alone was covered, leaving the head, or most of it, exposed. The results from such are, I think, wholly inconclusive. Stains, unless rubbed in with considerable force, were found to affect only the mucus covering the body, and to be removed with the discharge of this secretion.

Moreover, all of the methods employed were open to one serious objection: they injured and sooner or later killed the fish. Under such circumstances, it would be expected that disturbances of the normal reactions should occur, and such was indeed the case. A merely negative result in any instance, *i.e.*, the failure to respond to a given stimulus, cannot therefore be regarded as of great importance. We cannot, on the other hand, deny the significance of any positive results which were obtained, if the experiments were otherwise above criticism.

Suppose, now, that the anterior parts of a dark flounder be covered with a white mask, and that the animal be placed in a white tank. The fish would see itself as white. According to the hypothesis we are testing, there would seem to be no reason for change. The converse experiment might be performed with a pale

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[72] This suggestion of covering the fish with a cloth I owe to Professor Parker.

[73] Potassium permanganate and silver nitrate were the stains chiefly used. Both imparted a very dark shade to the skin. A white stain proved to be impracticable.

fish wearing a black mask and placed in a dark-walled tank. In this case, likewise, no change of pigmentation should occur, if a visual comparison between the animal's skin and the surrounding bottom is a necessary element in the reaction. Yet such changes did occur in a considerable number of my experiments, and in several cases they occurred under such circumstances as to go far, I believe, toward refuting the hypothesis in question.

In two such instances, where a white mask was employed, the fish (at first dark) did become pale upon a white bottom, and this reaction, in the case of each specimen, occurred again after a second trial.

Once more, a pale fish, which had been kept for ten days on white, was stained (anterior parts only) with potassium permanganate. This produced a continuous dark brown mask, covering as much of the animal's skin as it was enabled to see without bending the body. The fish, after return to the white box for a while, was later transferred to a black one. It became plainly darker in five hours and fairly dark in eight hours. The next day it died. Another specimen gave similar results, though not so well marked.

It must be allowed that in none of these cases was the shade assumed as pale (or as dark), as in normal specimens, but this I believe was due to the inhibitory effect of such severe treatment. That the latter is the true explanation is rendered probable by the fact that reactions were quite as likely to be inhibited which conformed to the requirements of the visual comparison hypothesis as were those which were contradictory to it. Thus one dark fish, which was covered with a *dark* mask and then transferred to white showed little or no change in the course of three days.

Fairness compels the mention of a case in which the reaction (in this instance to a white bottom) was almost wholly inhibited for two days by the presence of a white mask, but occurred within the next few hours after removal of the latter. In this case, the stitches and marginal portions of the cloth were left in situ, and it cannot be said that the effects of injury had been lessened by removing the other parts of the mask. This result, which was obtained much less conclusively with two other specimens, may be held to support the view that what the animal sees of its body is a deter-

mining factor in the reaction. I believe, however, that the inhibitory influence of the mask, in all these experiments, was due not only to the injury inflicted by needle and thread, but to the interference of the cloth with the respiratory movements of the operculum. This interference would of course cease with the removal of the overlying portion of the mask.

One test of the hypothesis in question, which was made unintentionally with Rhomboidichthys at Naples, must be referred to at this point, for I regard it as of greater significance than all of these experiments with artificial masks and stains. Specimen no. 10, which had been kept for fifteen or sixteen days in a marble-bottomed tank, and had in consequence assumed a high degree of pallor, was transferred to the coarse dark sand used in so many of my experiments. The fish immediately buried itself with great rapidity, and remained so, with only its eyes protruding, during its entire sojourn upon this bottom. It is probable that it never emerged (in the daytime, at least) except when forced to do so by myself, and at such times it concealed itself with extreme rapidity. Nevertheless, after two days, this specimen was nearly as dark as the sand, and after five days it was described in my notes as harmonizing almost perfectly with this material. After another extended sojourn (twenty-six days) upon white and pale gray backgrounds, the fish was placed upon the jet-black magnetite sand. In this, it displayed the same tendency toward concealment, remaining buried, except when forced to leave cover. Nevertheless, the fish was quite plainly darker after the lapse of a single day, and of a very dark shade after the lapse of six days (fig. 10d), although my notes state that "when placed here, the fish seemed almost white in comparison with the sand." There surely had been little opportunity in this case for the fish to observe the appearance of its own body.

The foregoing experiments with these two species of fish, although not free from contradictions, certainly do not bear out the visual comparison hypothesis, but rather come very near to refuting it altogether. A really satisfactory test of the alternate hypothesis seems likewise very difficult in practice, and, although I have devoted much time to the matter, I have, at the present writing,

no experimental results of interest to offer. The essential require-
ment for such a test would seem to be that the background to
which the fish is to cause its own shade to conform shall be illumi-
nated from a source of light independent of that which falls upon
the animal's eyes from overhead. Thus this background might be
made to appear dark or the contrary, relatively to the light which
seemed to illuminate it. A human observer, under similar cir-
cumstances, would be deceived, and would misjudge the shade
of the surface in question. Would not a fish do the same.[74]

After considerable experimenting, I believe that I have devised
an apparatus calculated to fulfil these conditions. This apparatus
I will not describe here, since I am not prepared to report upon
any results from its use. Owing to a lack of flounders of the right
species, the experiments must be deferred until the coming sum-
mer, when, I hope, it will be possible to settle the question at issue
by a few decisive tests.

SUMMARY AND CONCLUSIONS

Flounders of several species were found to undergo marked
changes in their color pattern or their general shade, when trans-
ferred from one type of bottom to another. The range and char-
acter of these pigment changes, and the nature of the stimuli
which provoked them, were subjected to considerable experimental
inquiry. The following are some of the principal facts which were
revealed through these investigations:

1. Fishes became very pale (in one species extremely so)
upon a white background; dark brown or nearly black upon a
black one, and of various intermediate shades upon bottoms of
gray, brown, etc.

2. The animals appeared to be limited in their capacity for
adjustment almost wholly to black, white, brown and gray tones.
Bright red or yellow backgrounds, for example, failed to call
forth adaptive responses, at least during periods quite sufficient

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[74] Again the spectre of 'anthropomorphism' may seem to rear its head. But
no. An optical illusion does not presuppose intelligent judgment (or misjudg-
ment), any more than does an ordinary normal perception.

for the other changes which are here described. In other words, the skin pigments which were displayed seemed to be restricted to components of the more habitual backgrounds encountered by such fishes.

3. Upon a homogeneous ground, the pigment of the skin was commonly much more uniformly distributed than upon a background having a diversified appearance.

4. Upon a mixed background, such as was afforded by one of the ordinary sands or gravels of its customary habitat, the fish took on a definite color pattern, which varied with the texture of the material, and was oftentimes in striking harmony with the latter.

5. Artificial backgrounds, containing variously distributed areas of pure black and white, called forth far more contrast in the skin patterns than did the less contrasted tones of the sand and gravel.

6. The principal markings constituting these various skin patterns were found to be permanent, in the sense that they always reappeared in the same positions, and even when the animal adapted itself to a homogeneous background, the outlines of most of these spots were still distinguishable. In the case of Rhomboidichthys podas, the arrangement of these spots was, in its essentials, constant for all members of the species. Regarding the other fishes used, I cannot speak with the same certainty.

7. The patterns assumed were consequently limited, in great degree, by fixed morphological conditions. Thus squares, cross-bands, circles, etc., were never copied in any true sense, by the fishes.

8. Within the limits thus imposed, the capacity of one of these species (Rhomboidichthys podas) to adapt itself in respect to the distribution of its skin pigments was often remarkable. For example, experiments with painted squares and circles of black and white showed that the resulting skin patterns depended not only upon the relative *amounts* of black and white in the background, but upon the *degree of subdivision* of the areas of the latter. As an example of this last point, when the background was divided into areas 2 millimeters square, a finer grained appearance was produced in the fish than when 1-centimeter squares were used.

9.   Thus, while the adaptation was most complete upon such backgrounds as formed a part of the natural habitat of the species, it was plainly not restricted to these cases, and the pigment was at times disposed in ways which, it seems likely, were quite foreign to the previous experience either of the individual or the race.   For example, the extremely pale, and perhaps also the very darkest conditions; likewise the vividly contrasted black-and-white condition, without intermediate shades, which was assumed by certain specimens upon some of the artificial backgrounds. Accordingly, the notion that the fish is limited to a few stereotyped responses, representing the most familiar types of habitat, must be rejected at once.

10.   Fishes of the same species differed greatly in their individual powers of adaptation, and some seemingly normal specimens possessed this power in a very limited degree.   Again the same fish acquired with practice (if this word may be allowed) the power of changing much more rapidly than before.   The time required for a radical change of shade or of pattern ranged from a fraction of a minute to several days.

11.   In the case of Rhomboidichthys, the underlying surface (more strictly, that part of the bottom immediately surrounding the fish) appeared to be the one chiefly effective in calling forth these changes.   The influence of the vertical walls of the vessel commonly seemed to be a subordinate one, even in cases where the fish was so large that it covered a considerable fraction of the bottom, and was obliged to lie constantly with its eyes close to one or another side of the jar.   Fairly conclusive evidence was offered, however, of the influence of the vertical walls of the latter, even upon this species. What the fish saw directly overhead seemed, on the contrary, to exert a negligible influence upon the color pattern.

12.   With the sand-dab, much clearer evidence was obtained of the influence of the vertical walls of the receptacle. These, at times, appeared to have an effect as great as, if not, indeed, greater than, that exerted by the bottom.   It must be noted here that this difference between the two species is perhaps to be attributed to the differing positions of their eyes.   Those of Rhomboidich-

thys are situated at the ends of movable stalks, so that this fish must be able to obtain a much nearer view of the bottom than is possible for Lophopsetta.

13. Within very wide limits, the degree of illumination of the background was found to have little or no effect upon the shade assumed by the fish. As a special example of this principle, fishes in a white receptacle, even when the latter was heavily shaded, became paler than fishes in a gray receptacle, even when this was exposed to bright light. In such cases, the dimly lighted white bottom of the one tank was actually darker than the brightly lighted gray bottom of the other, in the sense that the former reflected an absolutely smaller amount of light to the observer— whether human or piscine—than the latter. A rather curious problem was raised by a consideration of these facts, which was dealt with at some length and was made the object of special tests.

14. A specimen of Rhomboidichthys which was transferred while extremely pale to black sand, acquired a very dark shade, even though the fish remained persistently buried in this material, with only its eyes protruding. Again, specimens of Lophopsetta having their skin deeply stained, or wearing masks of cloth, were found, in some cases, to undergo pronounced adaptive changes, despite the fact that their body surface was disguised in this way. It is thus rendered highly improbable that any direct visual comparison on the part of the fish between its own body surface and the surrounding background is an essential factor in the production of these changes.

15. Fishes (Rhomboidichthys), when given the choice of two backgrounds, displayed no preference for the one which conformed more nearly to their own shade at the time. Likewise, specimens which were glaringly out of harmony with a given shade of sand appeared no more likely to conceal themselves beneath its surface than when their skin color was adjusted very closely to this.

16. When examined at night, after several hours of complete darkness, the fishes (Lophopsetta) were found to be in the same condition of pigmentation as when previously observed by daylight. Pale specimens, which were kept 5 to 7 days in a black-painted, light-proof box, became considerably darker during this

period, though remaining distinctly paler than dark control specimens with which they were compared. They acquired the same shade as the latter, however, after a few hours' exposure to light in the same box.

17. Experiments with fishes which had been deprived of their sight confirmed the findings of earlier investigators that the unimpaired functioning of at least one eye is necessary for the adjustment of the animal to its background. If blinded when in the dark condition, the fishes ordinarily remained dark, though they did not always permanently retain the darkest shade which is displayed by a normal specimen. If blinded when pale, they remained pale for about a day, but reverted to a darker condition, representing more nearly the resting state of the chromatophores. An interesting special case was discussed of fishes which had been adapted for a considerable period to a pale background, and afterwards for a brief period to a dark background. These reverted to the pale condition after blinding, though this later gave place once more to the dark state.

18. Destruction of the sight of one eye (whether the left or the right) had little or no effect upon the chromatic reactions of the majority of specimens of Lophopsetta.

19. Tactile stimuli, if effective at all, certainly played a quite subordinate part in evoking color changes of an adaptive nature, for the fishes responded as promptly to patterns painted upon the under side of strips of glass as to bottoms of stones and gravel whose complexity could be discerned by touch as well as by sight.

20. Very decided changes in the markings, as well as the general color-tone of the body were at times called forth by tactile or other non-visual stimuli, and the fish, when swimming, commonly presented a decidedly different aspect from that shown in the resting condition. But such changes as these belong to quite a different class from those which form the chief subject of the present paper.

Certain of the facts above summarized, deserve further discussion than was devoted to them in the body of the text.

We have seen that the skin of some of these fishes commonly assumes a nearly homogeneous tone upon a bottom of uniform color and shade, while presenting a more or less pronounced pattern upon a bottom of diversified appearance. Abbot Thayer[75] has pointed out that the breaking up of a uniform color tone by markings of any sort makes for concealment, and this is particularly true against a diversified background. This principle, without question, accounts for much of the effectiveness of the various patterns assumed by Rhomboidichthys and other flounders, and we must not be in too great haste to point out specific resemblances to particular backgrounds, merely because the fish ceases to be conspicuous upon these. I think, however, that a careful consideration of the experiments as a whole, and particularly of the facts referred to in paragraph 8 of the summary, forces us to the belief that there may be very specific relations between the distribution of light and shade in the background and the pigment pattern assumed by the fish.

Had we to do here merely with a general paling or darkening of the entire body surface, affecting spots and ground color to an equal extent, or even were there at the disposal of the fish one of two of these pigment patterns, corresponding to certain of the most frequent types of bottom, we might ascribe this power to a few comparatively simple reflexes. But we have seen that the responses are far from being as stereotyped as this. Certain areas become paler and others become darker, each more or less independently, and in varying degrees, depending upon the circumstances. At one time we have a large dark blotch covering a given portion of the surface; at another time, the pigment of this blotch has practically disappeared from view; at another yet this area has become broken up and diversified by the appearance of paler specks within it. Most of this change, too, is brought about by variations in the conspicuousness of groups of pigment cells

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[75] Popular Science Monthly, December, 1909; also book by Gerald Thayer entitled "Concealing Coloration in the Animal Kingdom. A Summary of Abbot H. Thayer's Discoveries," N. Y., 1909. It is likely that few biologists can follow Mr. Thayer in the unbridled zeal with which he strives to universalize this and the other important principles of animal coloration which he has discovered.

which never wholly fade from view. The pale specks which serve to "stipple" the dark blotches and give to them a fine-grained appearance (fig. 4a) may, in large part, be distinguished in the nearly solid blotches of the coarser pattern (fig. 4b).[76]

When we add to this complexity, the additional complexity due to differences of color proper (as distinguished from shade), it is difficult indeed to conceive of a nervous mechanism competent to bring about such changes. But conceivability is surely a poor criterion of possibility in biology, and we cannot see that a non-mechanical (i.e., vitalistic) interpretation of these phenomena would help us in the least. For, on the sensory and motor sides, this baffling complexity of mechanism would have to be granted in any case, and the only thing which the vitalist could do would be to posit a non-mechanical coordinating agency, which adapted the means to the end. But this, as has so often been pointed out, is a merely formal solution of the difficulty, and one totally impotent as a principle of scientific explanation.

That the stimuli which call forth these changes are visual rather than tactile has been shown, in my experiments, by the use of perfectly smooth glass plates, having the pattern painted upon the lower surface. That these stimuli are received through the eyes, rather than through the skin is, of course, not wholly proved by destroying the animal's sight, since the objection may always be raised that we have to do with inhibition through shock. On the other hand, the recent experiments of Parker[77] show pretty conclusively that the skin of at least some marine fishes is insensitive to light, even when the latter is of very high intensity. Were it proved, however, that such a general sensitiveness to light and shade was highly developed in the skin, it is impossible to see how responses to a *pattern* could be brought about through any organs except the eyes, for these alone are provided with the lenses necessary for the production of images.

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[76] As stated earlier (p. 416), the chromatophores themselves are probably distributed with much greater uniformity than the complexity of pattern would at first lead us to suppose. The position of the spots—actual and potential—may be largely determined by the position of the nerve termini.

[77] American Journal of Physiology, October, 1909. Fishes of nine species were used in Parker's experiments.

But aside from the evidence which they afford of the rôle played by the eyes in these *changes* of color, the blinding experiments seem to show that vision is necessary in order that the pigment cells shall remain in a given state of *tonus*, exception being made to the case of those fishes which are blinded in a uniformly dark state, representing most nearly the resting condition of the chromatophores. Continued adaptation to a less usual background, *e.g.*, a very pale one, may result in the new condition becoming more or less fixed. The latter may persist for a time after loss of sight, but the more habitual state of tonus finally reasserts itself. The cases mentioned at the close of section 17 of the summary might be explained by supposing that the pale condition had become in considerable measure fixed, so as to reappear after the stimuli responsible for the secondary dark condition had been withdrawn by destruction of the sight. The ultimate return to the dark state would be intelligible here as in the case of fishes which are blinded when pale. But if the foregoing interpretation is correct, it is hard to understand why any unusual state of the chromatophores which has but recently been acquired should not give place to the more habitual condition as soon as the light of day is withdrawn (*e.g.*, at night). But this was found not to be the case. Here, as so often happens, the simple and obvious explanation does not seem to contain the whole truth.

Evidence has been offered which seems to show conclusively that the plane in which a given surface lies with relation to the fish determines in some cases, whether or not it shall be effective in calling forth a given change. It was not made certain, however, that even in such cases, the matter was not decided by purely quantitative relations within the visual field. For, as was pointed out, we must distinguish between the potential and the actual visual fields. That the horizontal surface lying immediately about the fish is the one which is generally most potent in determining the reactions of Rhomboidichthys, might be due entirely to the fact that the animal's gaze is commonly turned in this direction. In experiments upon Lophopsetta, we found (p. 454) that when the bottom, plus the *upper* half of the vertical walls, were white, while the lower half of these walls was black,

the dark fishes which were used did not turn pale. On the other hand, the white bottom, plus the white *lower* half, or even lower fourth were found to call forth this change. These facts, which at first thought would seem to indicate the operation of some other factor than the relative amounts of black and white, do not in themselves force us to such a conclusion. If we assume that the animal's field of attention (due to the position of the eyes or otherwise) extends but little above the horizontal plane, the facts may, indeed, be explained on a purely quantitative basis. But this is probably not the whole truth. For it seems to follow from the considerations offered below that differences in the direction of different portions of the visual field probably condition the reactions of the fish in another important respect.

This problem is more clearly allied than might at first be supposed to another one which has received considerable attention in the present paper. I refer to the modus operandi of the stimuli which lead the fish to become very pale on a white surface and gray upon a gray surface, irrespective of the degree of illumination.[78] As I have already pointed out (probably quite needlessly), a surface of pure gray reflects white light, with no alteration except a diminution of its intensity. A human observer distinguishes a given object as gray, rather than white, only by reference to the degree of illumination to which it is subjected, and this last he infers from the appearance of the remainder of the visual field. Suppose, for example, that our visual field should for the moment consist of a single uniform surface, of which we had no prior knowledge, illuminated by a light of unknown intensity. Under such circumstances, we should be at a loss to say whether the surface was gray or white.[79] Once we have an idea of the degree of illumination, however, and we make the necessary correction for this, as, for example, when we view a piece of white paper in the twilight, we commonly pronounce it to be white, despite the abso-

[78] The reader, if interested in this part of the discussion, is advised to refer directly to the treatment of this question in the body of the text, particularly pp. 440–443 and 460–467. It would be impossible, without much undesirable repetition, for me to restate the entire problem here.

[79] Colors, of course, would still be distinguished.

lutely small amount of light reflected from it. In the same way, the interiors of the white vessels used in my experiments seemed to the outside observer[30] to be white, and those of the gray vessels to be gray, despite the fact that the latter actually appeared far lighter in a photograph.

Are the perceptions of the fish similarly determined? How can such a thing be possible, in cases where the uniform walls of the receptacle constitute practically the entire visual field of the animal? Without any outside standard of comparison, how can a heavily shaded surface of white appear paler than a brightly lighted surface of gray?

We have seen that one simple solution of this difficulty would be to assume that the fish makes a direct visual comparison between its own body surface and the bottom on which it lies. If the former is adjusted to the latter, the absolute degree of illumination which is common to the two is a matter of no possible consequence, for the object of this adjustment is the concealment of the animal. This hypothesis was, however, rejected, in view of pretty conclusive experimental evidence. Furthermore, it does not accord well with the fact (p. 443) that the behavior of the fish does not seem to be influenced in other ways by the color-phase in which the animal happens to be.

What, then, is the standard of comparison by which the fish (or its unconscious nervous mechanism, if the reader prefers) determines the shade to which the skin is to conform? In other words, if, as has been demonstrated, the absolute amount of light reflected from the background is not the only factor in the effective stimulus, what other one is there? As just stated, the human observer would decide the point by reference to other elements of the visual field. From the fish's point of view, the only other element of the visual field, besides the bottom and walls of its tank, is the illuminated area overhead, representing the source of light—commonly sunlight reflected from objects outside the tank. May not, then, the ratio between the light reflected from the near-

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[30] At least they did so to me. It is quite possible that one who did not appreciate the density of the shadow might have judged otherwise.

by surfaces within the tank and the light which enters the latter from above be that factor of the total stimulus which renders possible these accurate adjustments of the shade of the fish's body to that of its background?[81] I think that this is the true solution of the problem, and I hope, with apparatus already constructed, to put the question to experimental test, as soon as material is available.

That such a relation between the light intensities of two portions of the visual field may form an integral part of the immediate perception, without the necessity of rational mental processes, I think will be granted by all. Now naturally the fish knows nothing of the distinction between the source of the light and that part of the environment which is illuminated by it. There is for the animal but one continuous visual field, though this may not all be apprehended at once. The latter is constituted by various areas, differing in luminosity or in color. Those portions of this field which lie below, or but little above, a horizontal plane passing through the animal itself are the ones to which the appearance of the latter is adjusted. It is these which, as already seen, probably occupy the focus of attention most of the time. Those portions which lie more nearly overhead, and thus ordinarily beyond the focus of attention, must, however, serve in some way as a criterion by which the shade of the rest of the visual field is apprehended. With a given amount of light from outside the tank, a greater or a less amount of reflected light from the bottom would of course imply a lighter or a darker shade in the latter. On the other hand, with a given (absolute) amount of light reflected from the bottom, the occurrence of a low degree of illumination overhead would lead the animal to attribute a paler shade to this bottom (*i.e.,* *to see it as paler*) than if the source of light were a brilliant one.

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[81] These words, and in fact my entire discussion of this problem, down to the end of the next paragraph, were written before I had any knowledge of the almost identical hypothesis which was put forward some years ago by Keeble and Gamble (Phil. Trans. Roy. Soc., Series B, vol. 196, 1904). Under these circumstances, such a verbal coincidence as is to be noted in comparing their statement with mine is rather surprising. On p. 358, these authors state: ". . . on the white and black grounds the animal . . . appeals for pigment-guidance to the amount of light *scattered* or *absorbed* from the ground; or, as we put it previously, it is a reaction to the ratio $\frac{direct}{reflected}$ light."

Experiment alone (see p. 467) can place beyond question the accuracy of this interpretation. What is needed especially is a satisfactory determination of just which elements of the visual field it is to which the animal conforms its own appearance, and which ones it is that serve as a criterion by which the shade of the background is apprehended. So far as I can see, differences of *direction from the animal's eyes* are the only ones which can be invoked in differentiating these two sets of stimuli, and Keeble and Gamble (whose treatment of this problem was unknown to me when the foregoing discussion was written[82] incline to the same opinion. They hold (p. 354) that "in some way, the eye differentiates between the direct and the irregularly scattered light, in other words, it displays a certain dorsi-ventrality." Under ordinary conditions, the background is below and the source of light above. But the authors find that, if the conditions of illumination be artificially reversed, the "background" being above the animal and the light entering from below, the reaction to the former is the same as when it lies beneath them. Thus, they hold, "the dorsiventrality is probably not due to a permanent structural difference in the two sides of the eye." It is not clear, however, from their account of this experiment, that the conditions of illumination were not complicated by total reflection from the bottom of the jar. Unless the animals looked directly downward, or at least within a certain angle with the bottom, they would see, not a brightly lighted field below them, but the reflections of objects in the upper portions of the tank (pp. 427, 428 of the present paper).[83]

[82] See foot-note 81

[83] I cannot feel quite sure that the experiment of Bauer (Centralblatt für Physiologie, 1906), in which he used electric lights, placed above and below the glass container, is not open to the same objection. From this experiment and others, Bauer concluded that the assumption of a dark shade by *Idotea* was determined by "Simultankontrast," irrespective of the position of the contrasting portions of the visual field. This is certainly not true of fishes, as is shown by my experiments. (See particularly p. 451 et seq.)

The experiments of Mayerhofer, likewise, (op. cit., pp. 553, 554) in which a mirror was placed below the glass container, inclined at an angle of 45°, appear to me to be inconclusive, owing to the same apparent technical defect; and it is significant in this connection to note that fishes lighted from below, in this way, assumed the same shade as those kept in total darkness.

A word in regard to the utility of this power of color change in the life of the organism. Despite the recent reaction against extravagant applications of the protective coloration principle, it is difficult to doubt, in the present instance, either that this faculty has some use, or that it has been developed in some way because of its use. The end to be attained seems to be *concealment* and nothing else. No appeal to thermal regulation,[84] to possible "photoreceptive" or "photosynthetic" functions of the skin pigments, nor any other purely physiological explanation of the phenomena seems adequate. A complete explanation must regard ecological factors as well. Whether the utility of these changes to the fish consists primarily in their concealing the latter from its enemies or from its prey cannot, however, be stated without a greater familiarity with the bionomics of these species than the present writer possesses. I learn from several trustworthy observers that flounders of various kinds are preyed upon by sharks and other large fishes. The only information which I have relating directly to the enemies of any of the species which have been discussed in this paper, is the statement of Mr. Vinal Edwards that he has taken sand-dabs, along with other flounders, from the stomach of the cod. It is quite probable *a priori* that all of the species are similarly preyed upon.

As regard the prey of these fishes, I can but offer my own observation that specimens of Lophopsetta, when recently brought into the laboratory, frequently regurgitated the 'sand-launce' (Ammodytes americanus), sometimes in considerable numbers. It is not unlikely, therefore, that the cryptic coloration of flounders is of advantage in concealing them from smaller fishes[85] until the latter come within easy range.

These few meagre statements of course illustrate the paucity of our direct evidence upon the whole question of the utility of cryptic coloration, and indicate the inferential nature of most of our conclusions in this field.

February 23, 1911.

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[84] As suggested by Max Weber et al. (Van Rynberk, op. cit., p. 568 et seq.)
[85] Invertebrate food may perhaps be left out of consideration here.

EXPLANATION OF PLATES

The photographs, with the exception of 1*e* (by Dr. Victor Bauer), were all taken by the author. The water-color sketches (plate 6) are the work of Mr. V. Serino, an artist in the employ of the Naples Station.

The figures all relate to a single species, Rhomboidichthys podas (Delaroche), and all are reduced to approximately one half the natural size.

Figures bearing the same number represent different views of the same fish. These are arranged with a view to easy comparison. Thus the order in which the views of a single specimen are presented does not necessarily bear any relation to the order in which the corresponding changes were undergone in the course of the experiments.

For an account of the photographic methods employed see pp. 418–421 of the text.

PLATE 1

EXPLANATION OF FIGURES

Views of specimen no. 1: *a*, on a dark, mixed sand (after one day); *b*, on fine gravel (after one day); *c*, on fine jet-black (magnetite) sand (after four days); *d*, on a very coarse reddish sand (after eight days); *e*, on a coarse gravel, devoid of sand (after two days).

Views of specimen no. 1: f, on painted squares, 2 cm. sq. (after four days); g, do., $4\frac{1}{2}$ cm. sq. (after seven days); h, do., 1 cm. sq. (after four days); i, do., 2 mm. sq. (after one day).

Views of specimen no. 1: j, after three days on background figured; k, after fourteen days on white marble bottom (the fish was in reality much paler than the photograph would seem to indicate); l, after three days on the background figured; m, after six days on the background figured.

PLATE 4

Views of specimen no. 2: *a*, on dark mixed sand, partly covered with this material (after two days); *b*, on white glass plate, shortly after transfer to this following sojourn on dark sand; *c*, on coarse gravel (after two days); *d*, on white glass plate, shortly after transfer to this, following sojourn on coarse gravel. (Compare this with *b*. In *d*, the gravel pattern has persisted to some degree, despite an immediate partial disappearance of this).

2 a

2 b

2 c

2 d

PLATE 5

Views of specimen no. 2: *e*, upon fine gravel (after two or three days); *f*, upon dark, mixed sand (after two days).

Views of specimen no. 3: *a*, after one day on present ground; *b*, taken on the day when first placed on this sand. (Note the inferior power of adaptation shown by this fish, as compared with the last. The harmony with the backgrounds increased little if any beyond the condition shown in the photographs).

PLATE 6

Views of specimen no. 3, in the 'sand' and 'gravel' phases. Unfortunately, the capacity of this specimen for such adaptations proved to be comparatively small.

3 c

3 d

V. Serino, del.

Views of specimen no. 4: *a*, after six days on pattern shown; *b*, after three days on pattern shown (compare minutely with last); *c*, after nine days on the dark sand; *d*, after three days on the pattern shown; *e*, after three days on the pattern shown.
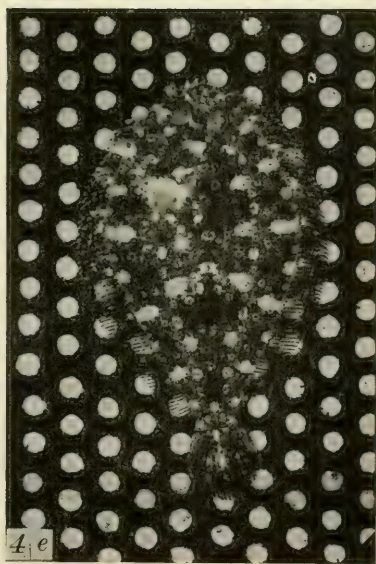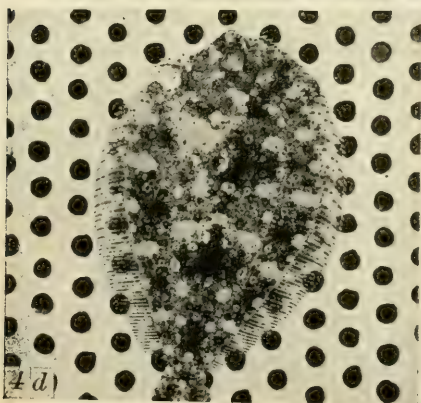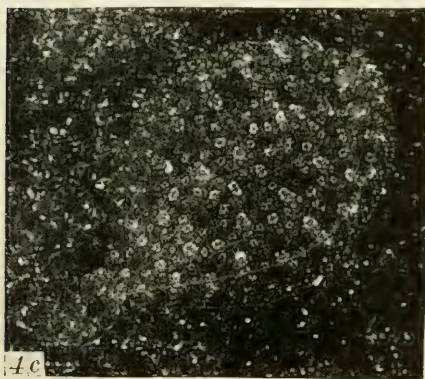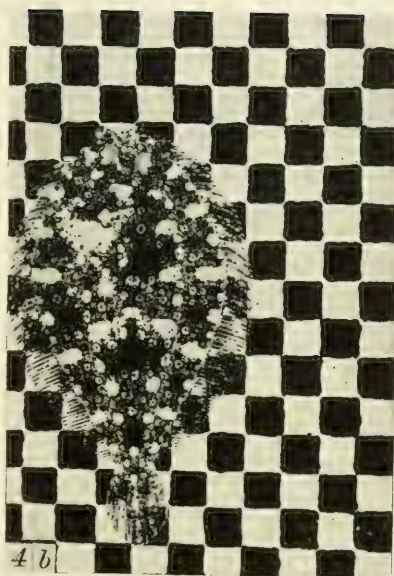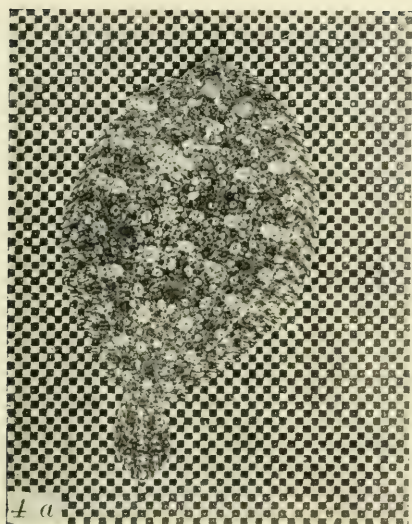
Views of specimen no. 4: f, after seven days on present background (condition of rest); g, same, when preparing to swim; h, after one day on present background; i, after three days on a background of gray, of a shade approximately matching that produced on a color-wheel by the use of two parts of black and one of white. (The harmony between the fish and the bottom was really much greater than would seem to be the case from this photograph, which has made the fish seem darker).

Specimen no. 5: view inserted to show a particularly striking gravel pattern (taken after eight days). (There are in reality a few particles of gravel on the back of the fish).
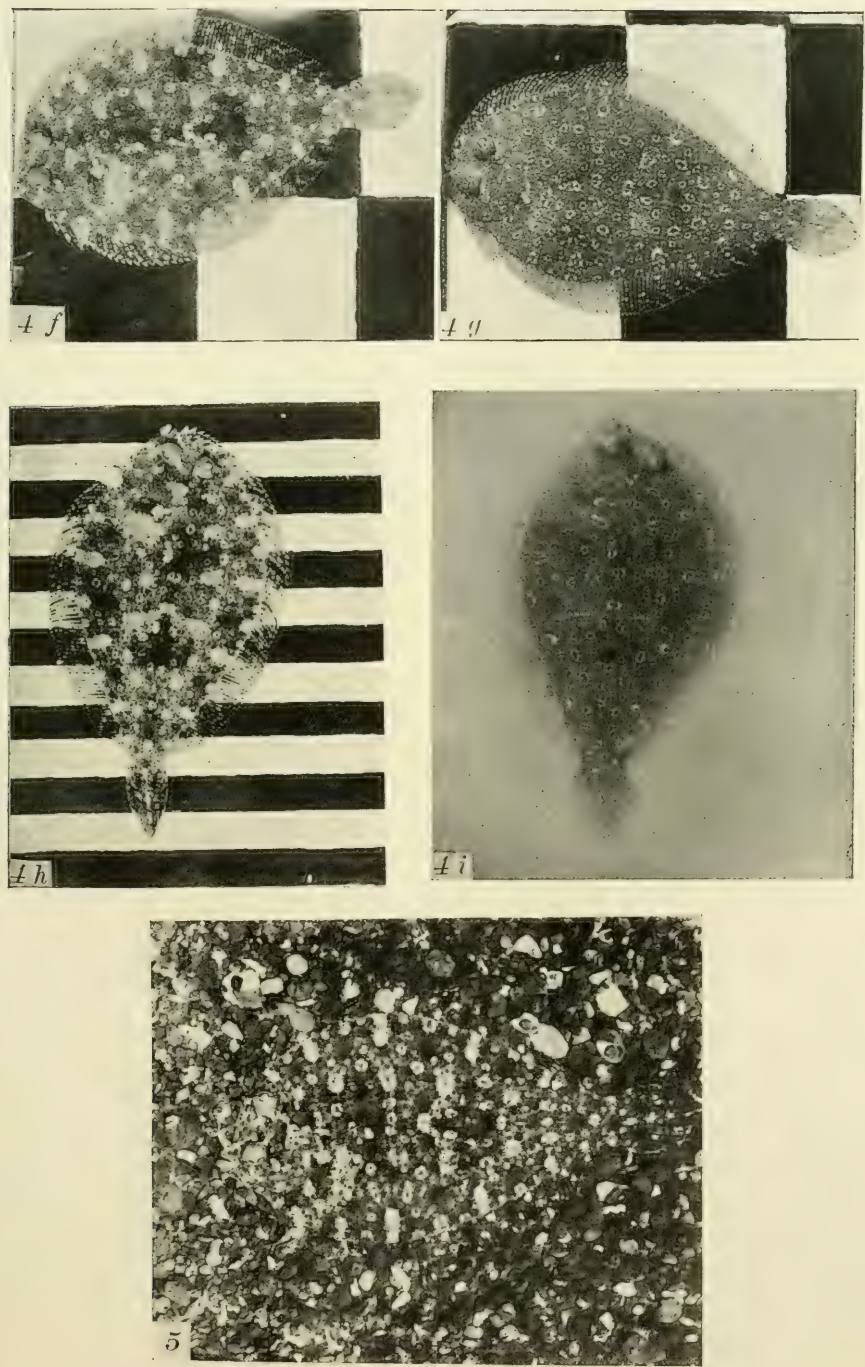
Views of specimen no. 6: *a*, upon dark, mixed sand (after one day); *b*, upon the same sand, with the addition of white pebbles (after three days); *c*, on coarse, reddish sand (after six days).

Specimen no. 5: showing conspicuously mottled appearance, unusual in a specimen just brought to the laboratory.

6 a

6 b

6 c

7
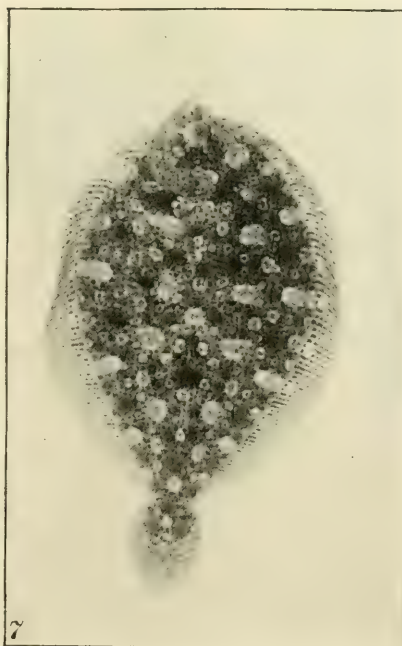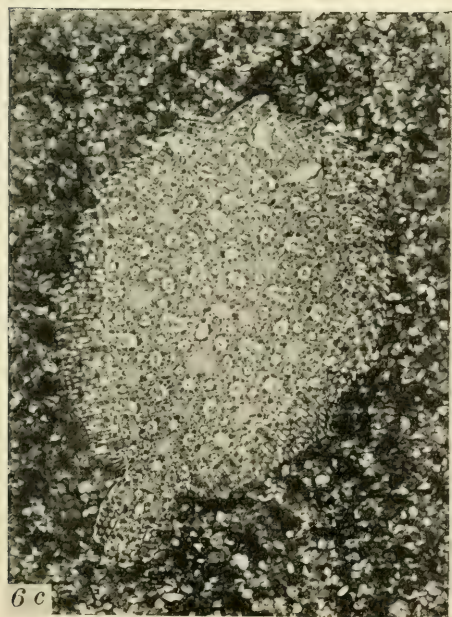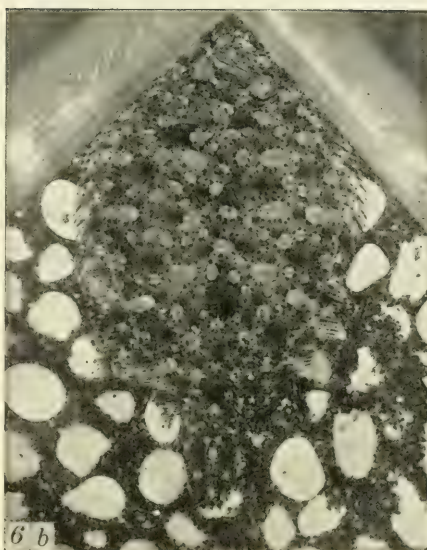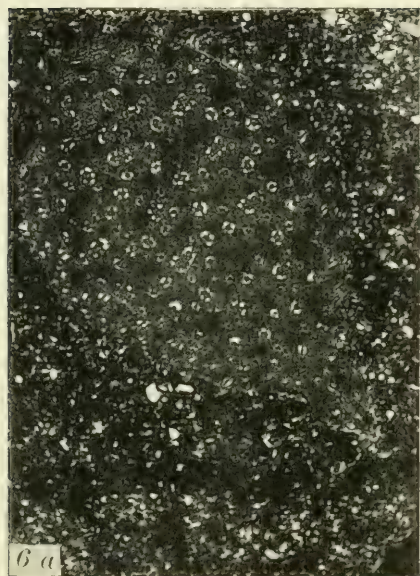
Views of specimen no. 8: *a*, after four days on material shown; *b*, in jar having black bottom, and transparent walls, surrounded by gravel (after seven days); *c*, in jar having black walls and transparent bottom, with gravel underneath (after ten days); *d*, taken on a black bottom, immediately after transfer from a white-bottomed, black-walled jar, in which the fish had remained 9 days.
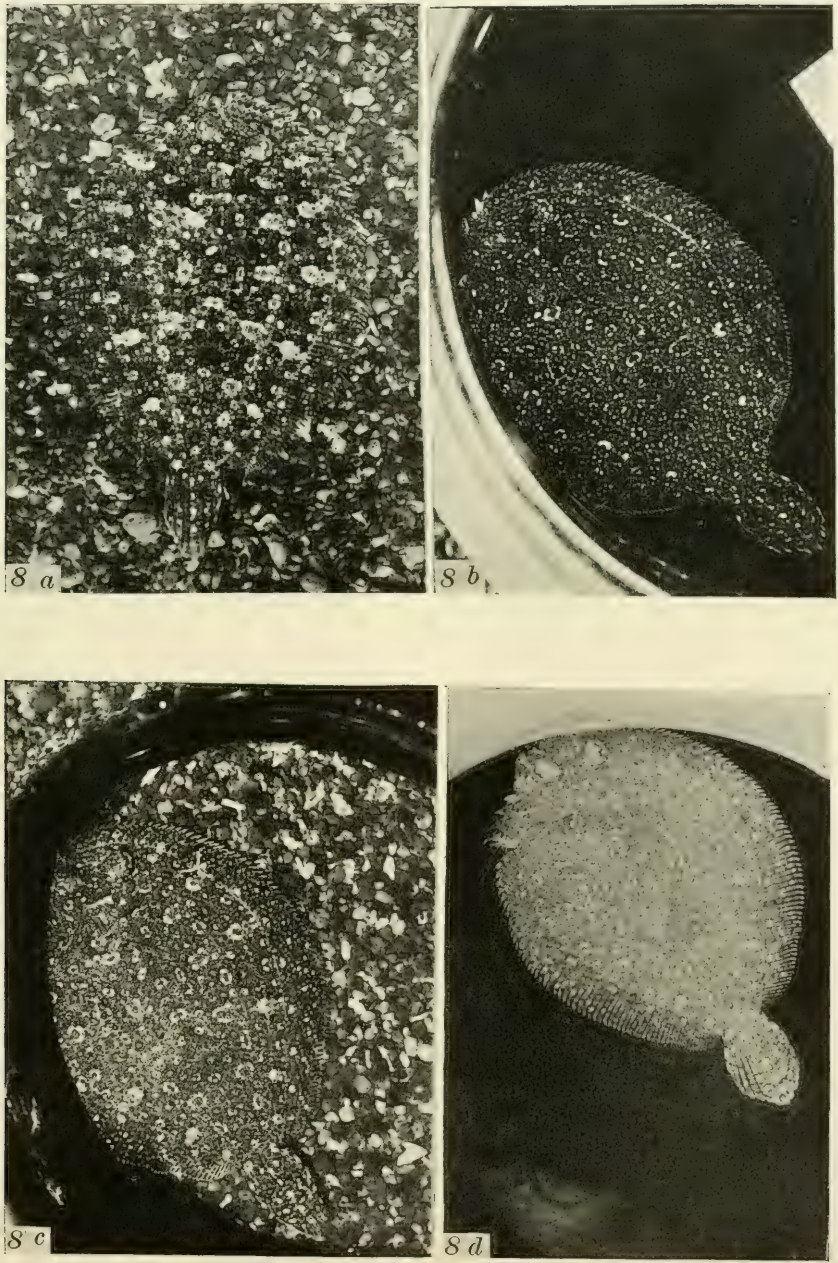
Views of specimen no. 9: *a*, on dark, mixed sand (after two days); *b*, in jar having black walls and gravel bottom (after two days); *c*, in jar having white walls and black bottom (after five days); *d*, in jar having black walls and black bottom (after one day); (Comparison between the last two shows plainly the effect of the white walls in the former case).

9 a

9 b

9 c

9 d
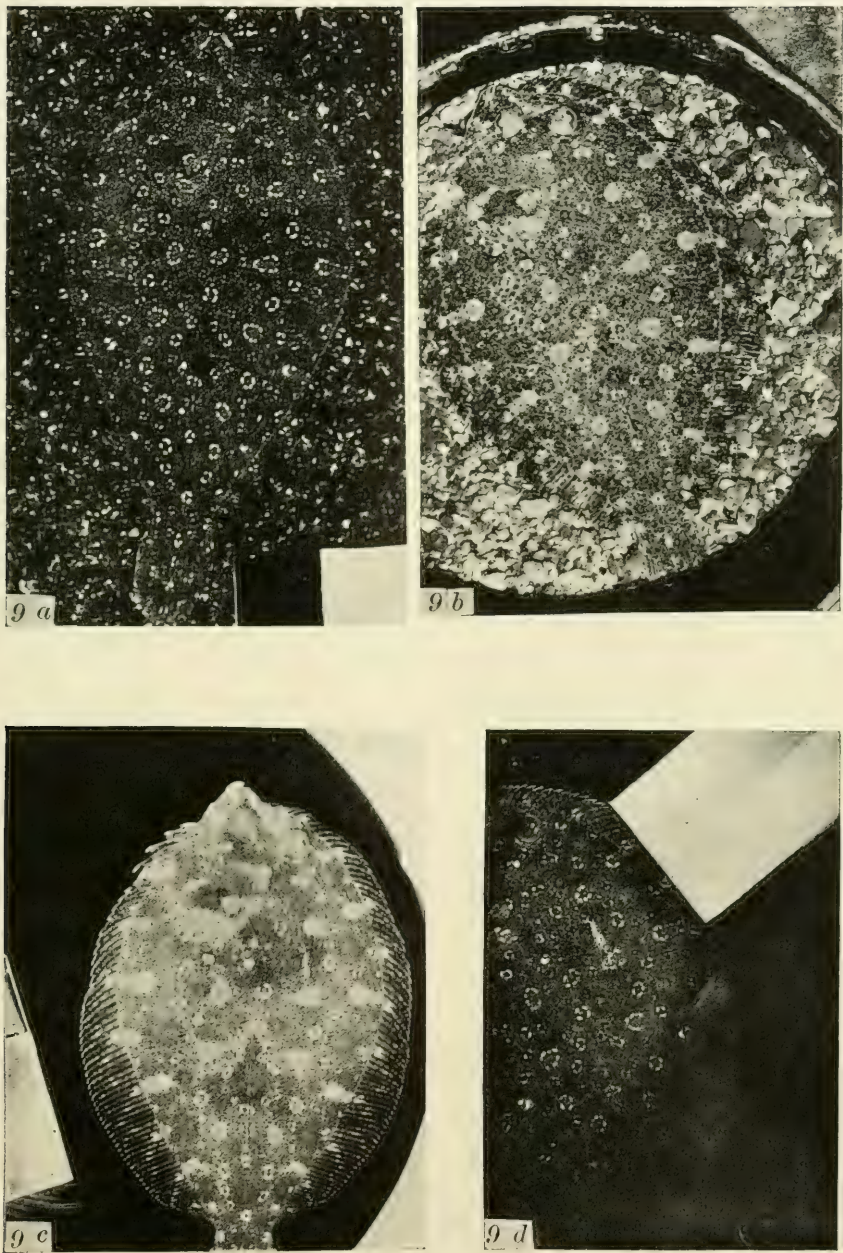
PLATE 12

10 *a* After fourteen days on white marble bottom.

11 *a* After fifteen days on white marble bottom. (Both of these fishes appear much too dark).

11 *b* Showing possible effects of black specks in the white field.

10 *b*–11 *c* Showing condition of former fish, after thirteen days on the dark sand as compared with the latter, which had just been transferred to this.

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10 *e* After blinding, during recently acquired dark condition, following long sojourn on pale bottoms. The result is a return to the pale phase.

11 *e* After eleven days on jet-black sand
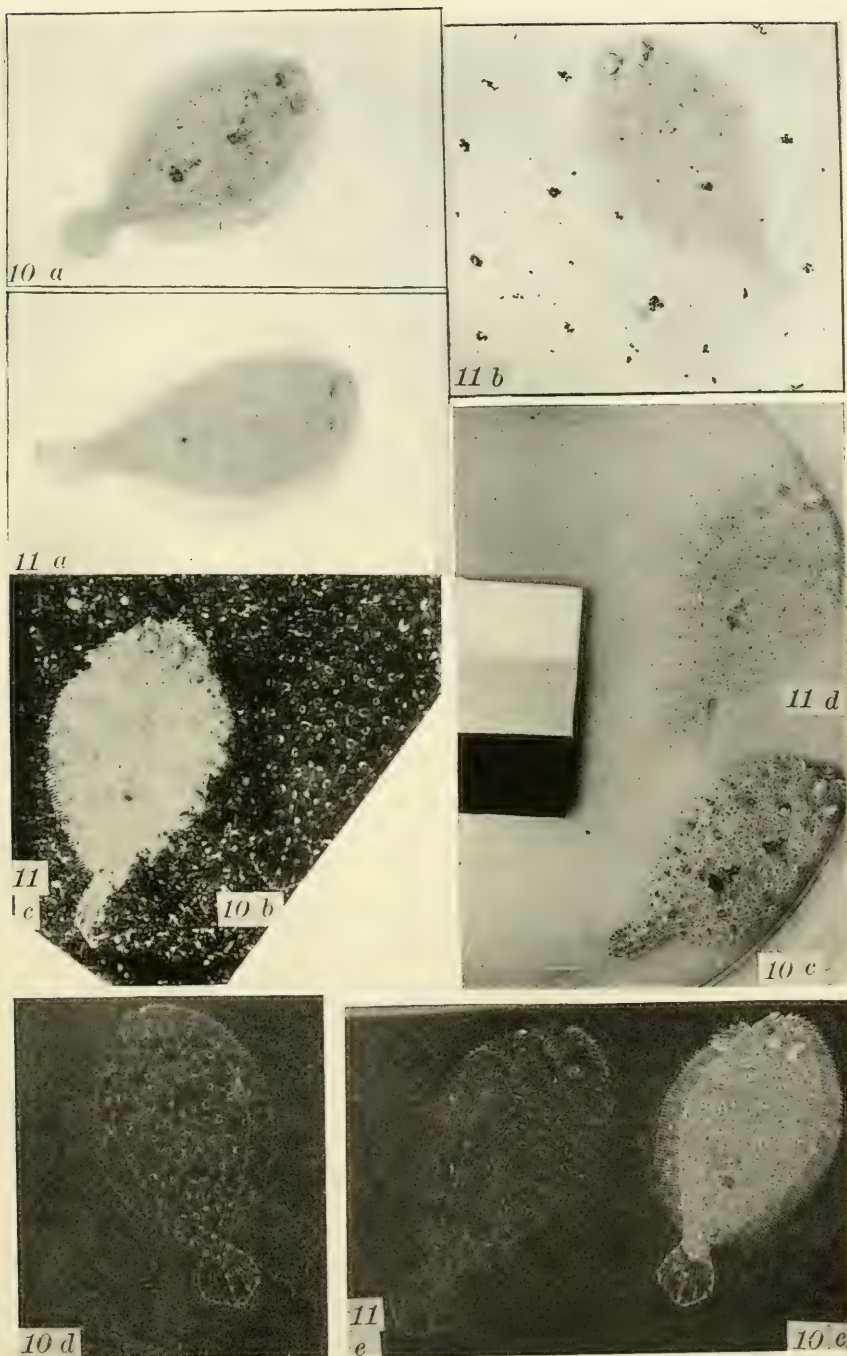
12 *a* Against background of gray (same gray as in 4 *i*), immediately after transfer from dark sand.

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11 *h*–12 *d* Appearance of the same two fishes, four days after the reversal of the above conditions (11 being in white jar, 12 in gray). (The reader must not be misled by the altered positions of the two fishes in the later picture. The identity of each specimen is revealed by its size, no. 12 being the larger).

12 a

12 b

11 f

12 c

11 g

11 h

12 d

STUDIES ON THE PERMEABILITY OF CELLS

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THREE FIGURES

CONTENTS

INTRODUCTION

1. Historical

Within the past fifty years a great deal of scientific research has been directed toward advancing our knowledge of the manner in which substances may pass into living cells and of the classes of substances which may or may not enter. Intimately connected with the question of the permeability of cells are the phenomena arising from the existence of an osmotic pressure without and within the cell, and the rigidity or turgor of plant parts directly dependent on the latter. This relation is most evident when we recall that the existence of a continual turgor of plant structures depends on the possession, by the individual cells, of a surface membrane which prevents the diffusion outward of most of the substances dissolved in their sap vacuoles.

Questions concerning osmotic pressure must therefore always go hand in hand with questions concerning the permeability of the membrane whose presence is one condition for the existence of that pressure. Historically the two subjects have developed simultaneously.

It is hardly within the scope of this paper to give a detailed account of the history of this complicated subject, so numerous are the papers dealing with its different phases. The fundamental facts were outlined by three observers during the latter half of the last century. Their influence has been so great that I shall mention briefly the contribution of each.

Nägeli ('55) first investigated the osmotic properties of the cell and made clear the cause of turgor and of plasmolysis. The word plasmolysis was introduced later by DeVries. It is to Pfeffer ('77, '86, '90) and DeVries, ('71, '77, '84, '85) however, that we owe our present conception of the important rôle played by diffusion and osmotic pressure. Both of these authors investigated the magnitude of the pressures existing in plant cells and the properties of the cell membranes. Pfeffer has especially emphasized the conditions under which accumulation of substances takes place; DeVries the importance of the vacuolar

membrane, designated by him the tonoplast. The generalizations made by these three botanists, in which the discovery of semipermeable precipitation membranes by M. Traube ('67) has played a most important part, have been extended and confirmed by a host of recent experimenters, Overton, Hoeber, Nathanson, Ruhland, Hamburger, R. Lillie, Koeppe, Gryns, Hedin, Asher, J. Loeb, and many others. Although most studies on permeability have been carried out on plant cells, the same essential relations are exhibited by animal cells.

2. Theories concerning permeability

The more recent studies, especially those of Overton ('95, '97, '99, '00), have been concerned with the classes of substances which may or may not pass the plasma membrane. This at once raises two important questions.

1. How does a substance enter?

2. What is the nature of the cell surface or plasma membrane?

In answer to each of these questions several theories have been advanced. Let us consider first the nature of the plasma membrane.

Quincke ('88), in order to account for the power of movement of amoeboid cells as well as certain peculiar osmotic properties, assumed that a thin film of oil was present at the surface. Overton has explained the very rapid entrance of ether and fat-soluble substances by assuming that the plasma membrane is composed largely of lipoids like lecithin. This view was later modified to explain the entrance of substances insoluble in lipoids by regarding the cell surface as a mosaic of proteid plus cholesterin (Nathanson, '04, a) or proteid plus lecithin. On the other hand Pfeffer has always insisted that the membrane is chiefly proteid, while Robertson ('08) considers it a form of modified protein comparable to that which remains about droplets of chloroform shaken up with protein solutions and then washed repeatedly in water.

Three general theories, based on Van't Hoff's view of the driving power in osmosis, have been advanced to account for the passage of substances through membranes:—the filter theory, the solution theory, and the chemical combination theory. The filter theory regards a membrane as a molecular sieve. Whether it is permeable to a given substance will depend on the relative sizes of the molecules of the substance and the interstices of the membrane. A study of artificial precipitation membranes has afforded considerable evidence against such a simple explanation. According to the second theory a substance must dissolve in the membrane in order to pass through, and according to the last theory the substance must combine with the membrane before it may pass.

J. Traube, ('04, '08, '09, '10) on the other hand, regards the driving force in osmosis, as an 'Oberflächendruck,' later called 'Haftdruck,' measured by the tendency of the substance to increase or decrease the surface tension of the solvent. The membrane separating two phases is not the important thing but the difference in surface tensions, the phase of lowest surface tension tending to pass through the membrane into that of greater surface tension. Traube admits that the Haftdruck of the membrane may also be a determining factor ('10, p. 533).

At present there are two general views as to the classes of substances which may diffuse into cells. These are based largely on conceptions regarding the nature of the cell membrane and the physical or chemical process by which a substance may pass it. The results obtained by different methods of investigation and their interpretation have been in many cases conflicting.

Inasmuch as my studies on the permeability of cells show in the clearest manner the existence of two distinct classes of alkalies with respect to their ability to enter, a brief statement of the opposed views may not be out of place.

Overton and Hoeber classify substances into lipoid-soluble and lipoid-insoluble. The former are found to enter cells very rapidly, the latter not at all when tested by the plasmolytic method (p. 512). Yet the lipoid-insoluble substances are just those which we know by analysis and microchemical tests to

occur in cells (inorganic salts, sugars, etc.).   Further, the entrance of KNO_3 can be demonstrated by the diphenylamine reaction, while the cell remains plasmolysed.   Consequently Overton and Hoeber assumed that cells have a physical and a physiological permeability.   The lipoid soluble substances enter in a purely physical manner, by simple diffusion, involving solution in the lipoids of which the plasma membrane is assumed to be composed.   The lipoid-insoluble substances enter in some other way, but not by diffusion.   As evidence of this Overton calls attention to the fact that the neutral salts, sugars, etc., are just the substances which are known to pass into cells or through cells from regions of lower to regions of higher concentration without chemical change.   Some other factor than diffusion must be involved.[1]   Hoeber ('09) now admits that the lipoid theory does not hold for dyes and that it must undergo more or less remodelling (p. 78).

On the other hand, Overton's opponents draw no distinction between lipoid-soluble and lipoid-insoluble substances, but regard the entrance of any solute as a process of simple diffusion, (without considering how the substance passes the membrane), the only difference lying in the relative rates of diffusion.

The presence of salts in cells in different proportions from the medium is explained as due to combination with proteids in the cell.   Indeed, Moore and Roaf ('07) have gone so far as to deny any importance to the existence of a surface membrane in regulating the entrance of salts into red blood corpuscles, but regard the difference in salt content between cell and medium as entirely due to formation of salt proteid compounds.

A great deal of confusion has arisen from the fact that a cell may change in permeability from time to time.   Differences are

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[1] It would seem that we must draw the distinction between the accumulation of salts in solution in vacuoles and the existence of salts (as determined by chemical analysis) in the protoplasm of cells without such structures.   The former is a phenomenon comparable to the passage of NaCl through loops of intestine from dilute to more concentrated solution.   Two bounding surfaces are involved. The latter is possibly explainable by the formation of ion-proteid compounds within the cell as developed by Moore and Roaf (Biochemical Journal, Vol. 3, p. 55, 1907) to account for the high K content of the red blood corpuscle.

to be noted especially between rest and activity, due to stimula-
tion. A further source of error results from the fact that the
very substance whose penetration we are studying may change
the permeability of the cell in the concentrations used. This
naturally leads to a brief consideration of the methods of test-
ing permeability used by various authors. They may be con-
veniently classified as follows:

3. Methods of testing permeability

1. Plasmolytic or osmometric (depending on changes in
volume and turgor of the cell).

a. Direct observation of plasmolysis (DeVries, Overton).

b. Indirect, by noting cessation of movement in motile bac-
teria (Wladimiroff, Zeit. f. physik. Chem. 7 p. 527, 1891).

c. Indirect, by weighing (Overton, Pflüger's Archiv. 92, p. 115,
1902, Loeb, Pflüger's Archiv. 69, p. 1, 1897 and E. Cooke, Jour-
nal of Physiology, 23, p. 137, 1898).

d. Indirect, by noting liberation of haemoglobin in isotonic
permeating solutions (Gryns, Pflüger's Archiv. 63, p. 86, 1896).

e. Indirect, by determining change in volume of centrifuged
corpuscles (Koeppe, Gryns, Hedin).

f. Indirect, by determining the concentration in sea water and
the concentration, pure, capable of causing artificial partheno-
genesis (Loeb. Univ. of Calif. Pub. 3, p. 81, 1908)

2. Observational *a.* Directly, as the entrance of dyes (Pfeffer,
Overton, Hoeber, Ruhland).

b. Introduction of an indicator (as neutral red) and subse-
quent change.

c. By some change produced in a substance (as tannin) already
present in the cell (Overton, Zeit. Physik. Chem. 22, p. 189, 1897).

d. By microchemical tests (as the determination of KNO_3 by
diphenylamin; Molish).

3. Analytical (chemical or otherwise) *a.* By analysis of cells themselves.

b. By analysis of medium (chemically, or by determining freezing point or electrical conductivity) after diffusion into the cell has taken place.

c. By analysis of the medium after diffusion from the cell has taken place.

4. Conductivity [of blood corpuscles (Stewart, Tangl and Burgarszky, and Roth) or of eggs (McClendon)].

5. Alteration of function (on the assumption that to affect a cell the substance must enter).

a. Test by toxicity.

b. Test by narcosis (Overton).

c. Test by change in manner of response to stimuli (Loeb, Dynamics of Living Matter, p. 842, New York, 1906).

d. Test of effectiveness in causing artificial parthenogenesis (Loeb, J., Chemische Konstitution und physiologische Wirksamkeit von Sauren. Biochemische Zeitschrift, 15, p. 254, 1909).

No one of the above methods can be claimed as universally better than any of the others. Recent researches have exposed sources of error in the application of the plasmolytic method (Osterhaut, '08). Mass analyses of cells give us no hint as to the location of the substance in the cell or the state in which it is present, in solution or in combination.

The evidence (pp. 543–546) in the case of the inorganic hydroxides shows that these alkalies may produce functional changes and death of Paramoecium without entering in sufficient quantity to affect granules stained in neutral red within the cell, and its effect must be on the membrane. Hoeber regards the surface of the cell as the point of attack of the strongly dissociated substances in general. Only after the cell surface has been fundamentally modified does the reagent pass in.

Whenever applicable the observational methods are most useful in studying problems in permeability, for they not only answer the general question concerning penetration of the substance in question, but also enable us to locate the reagent in the cell and

to determine changes in function associated with the entrance of a given quantity.

Most of the present experiments were performed in the Zoölogical Laboratory of Columbia University. The study of marine eggs was made possible by a visit to the Tortugas Laboratory of the Carnegie Institution and the Marine Biological Laboratory at Woods Hole. I wish to express my indebtedness to Dr. Mayer for the many special opportunities for research offered me at the former station and to Dr. Morgan for permission to use a Columbia table at Woods Hole as well as for many helpful suggestions.

THE PENETRATION OF ANILINE DYES

1. *Mechanism of staining plant cells*

In studying permeability for alkalies, to be discussed below, penetration was indicated by the change in color of neutral red in which the cells had been stained. In order to determine under what conditions neutral red exists in the cell and the nature of the dye compounds which the alkali must decompose on entering, I have conducted a few experiments on certain dyes with the above points in mind. The most interesting fact obtained is that basic dyes as a rule cannot enter cells in the presence of a trace of acid in the medium whereas certain acid dyes do not enter cells in neutral or weakly alkaline solution but readily stain and kill the cell in weakly acid solution. We should naturally suppose the explanation of the above results to be in the effect of the acid or alkali on the dissociation of the dye molecules.

a. Overton's hypothesis:—Overton had at one time supposed that only the free dye base of basic dyes might enter cells. Since basic dyes are combination of weak bases with strong acids we should expect them to be hydrolytically dissociated in water, thus:

$$RCl + H_2O = ROH + HCl$$

Only the ROH and not the RCl might enter. Overton ('00) later abandoned this idea since he was unable to show that the dye acetates entered the cell more readily than the dye chlorides.

The combination of a weak base and a weak acid undergoes a greater hydrolytic splitting, more of the free dye base is produced, and the cell should stain more rapidly in the acetate. But such was not found to be the case.

The addition of a slight amount of acid will prevent the hydrolytic dissociation and this test must be a surer one than Overton's. I have consequently come to the conclusion that Overton's first hypothesis is the correct one. The detailed evidence for this is given below. The dyes used are mostly the same that Overton experimented with belonging to the triphenyl-methane and chinonimid groups.

b. Elodea:—Pfeffer ('86) first studied the absorption of aniline stains and gave us a clear account of the mechanism of accumulation. The dyes collect as granular colored tannin precipitates in Spirogyra. The case of Elodea is different. Neutral red is not typically precipitated, but collects in solution of a red color.[2] It likewise collects as a red solution in the vacuole from alkaline tap water of a pale yellow color. This suggested that the sap vacuole is slightly acid and in the acid condition neutral red cannot pass out. Consequently it is slowly accumulated. (Harvey '10). I was thus led to try staining in weakly acid solution. While it is true that neutral red does not enter from the HCl or CH_3COOH acid condition, the acid present in Elodea is probably a very complex organic one. I have been unable to detect any red coloration by crushing the leaves on blue litmus paper.

The following experiment shows that the dye will not pass into the cell in the acid condition. Elodea leaves were placed in each of the following solutions:
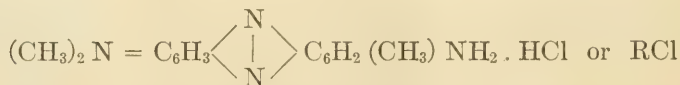
A. 50 cc. tap water + 1 drop 0.1 per cent neutral red.
B. 50 cc. 0.001 n HCL + 1 drop 0.1 per cent neutral red.
C. 50 cc. 0.001 n NaOH + 1 drop 0.1 per cent neutral red.

After six hours the leaf cells in A and C were found to have accumulated large quantities of the dye while the cells of B contain no dye at all. Leaves in B are not injured for protoplasmic

[2] In some cells red globules may be seen; In others red needle crystals. In alkaline condition neutral red is yellow; in neutral and acid condition, red.

rotation goes on and when placed in A they were able to collect the dyestuff from the surrounding solution. The stain may be 'adsorbed' by the cellulose cell walls from acid solutions just as it is adsorbed by glass only when in the acid condition. But this phenomenon has nothing to do with the question of the permeability of the cells for the dye, although on casual observation the appearance of dye accumulation may be given. Elodea cells fail also to stain in $\frac{N}{2000}$ acetic acid, yet accumulate the dye if transferred to a neutral solution.[3]

    c. Theory of indicators applied: Indicators as neutral red or litmus are very weak bases or acids. According to Ostwald, the color change is due to the transformation of the acid or base into a salt which is very highly ionized. The ions give the color to the solution and their color is different from that of the undissociated molecule of the free color base or acid, only slightly ionized in solution. Neutral red has the following structure:

$$(CH_3)_2\,N = C_6H_3\!\!\left\langle\!\!\begin{array}{c} N \\ | \\ N \end{array}\!\!\right\rangle\!\!C_6H_2\,(CH_3)\,NH_2\,.\,HCl \ \ or \ \ RCl$$

A small amount of acid converts all of the neutral red into dissociated $R+$ and $Cl-$. A small amount of alkali forms ROH, undissociated, and it is the undissociated color base which may enter cells. In the neutral condition a small proportion of free base ROH is present, due to hydrolytic dissociation. Consequently cells may be stained in neutral solution.

    d. Action of the acid is on the dye: That the effect of the acid is on the dye and not on the plasma membrane of the cells, decreasing its permeability, is made probable by the following facts.

    1. Certain acid dyes (eosin) enter only in the presence of dilute acid and fail to enter in alkaline solution.

    2. The presence of dilute HCl does not prevent the toxic effect of heavy metal salts like $CuCl_2$.

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   [3] The observations refer only to the normal rotating cells and not to certain large cells filled with a mass of white granular matter, which stains in acid solution.

3. The relations of basic dyes are exactly comparable to those found by Overton ('97) for certain alkaloids which are weak bases, and do not enter cells in acid solution. In the acid an alkaloid salt is of course formed.

I have observed that caffein, on the contrary, enters equally well in acid, neutral, and alkaline solution. It was compared with strychnin in similar percentage concentrations:

Spirogyra cells were placed in the following solutions:

A. 0.01 per cent strychnin sulphate in $\frac{N}{750}$ NaOH tap water.
B. 0.01 per cent strychnin sulphate in $\frac{N}{750}$ HCl tap water.
C. 0.01 per cent strychnin sulphate in tap water.
D. 0.0125 per cent caffein in $\frac{N}{750}$ NaOH tap water.
E. 0.0125 per cent caffein in $\frac{N}{750}$ HCl tap water.
F. 0.0125 per cent caffein in tap water.
G. $\frac{N}{750}$ NaOH tap water.
H. $\frac{N}{750}$ HCl tap water.

In A the strychnin passes into the cells and forms a granular reticulum and within twenty minutes the cells have lost their turgor and secrete a sticky substance. In C strychnin also enters but less rapidly and death results after about one hour. In B no strychnin precipitate is formed and the cells are normal after one hour.

In D, E, and F, an equal amount of caffein precipitate is formed after 45 minutes in each case.

The cells in G and H are quite unaffected by the acid or alkali after one hour.

e. Spirogyra: Exactly the same permeability relations hold for Spirogyra, sea urchin and starfish eggs, and Paramoecium. The mechanism of accumulation is different in each case. In the study of dyes we have always to consider two points—in what condition the dye enters and by what means it is made visible within. There must always be an accumulation of some kind for otherwise the color would not be apparent in so small a layer.

I have also studied the entrance of several others dyes in the acid and alkaline condition into Spirogyra and the same general law appears to hold. The results are best given in the form of a table which does not pretend to be exhaustive, but simply to

show that the conditions determining absorption of neutral red are equally true of other classes of dye stuffs (table 1).

Besides Pfeffer's original monograph, the most important comparative studies of permeability for the aniline dyes have been made by Overton, Ruhland, and Hoeber and Robertson.

Overton ('00) studied the permeability of both plant and animal cells in neutral solution for many different dyes and the solubility of the same dyes in olive oil and mixtures of lipoids. He came to the conclusion that the lipoid-soluble dyes enter living cells and the lipoid-insoluble do not. It has since been found that there are certain exceptions to Overton's conclusion.

Ruhland ('08) pointed out that there are some dyes which are lipoid soluble and fail to enter living cells, others are lipoid insoluble yet enter readily and still others which may enter, yet show no relation between rate of entrance and solubility in lipoids.

Hoeber's ('09) recent study of the same question gave a similar result to that of Ruhland with respect to certain dyes. He concludes that the facts correspond better with the 'Satz' that basic dye stuffs are *intra vitam* stains and acid dye stuffs are not.

Robertson ('08) has attacked Overton's original position by a study of the partition coefficient of various analine dyes in ethylacetate, ethylbenzoate, triacetin and triolein. He came to the conclusion that the solution of an acid dye in fatty substances is increased by the addition of acid, the basic by the addition of an alkali. In other words the free color acids or bases are more soluble in lipoids than their salts. Robertson also studied the stainability of fat cells, connective tisue cells, and red blood corpuscles (fixed on a slide) in acid and alkaline solution. But the HCl and NaOH used were so strong ($\frac{N}{100}$) that they must have killed the cells and no conclusions as to the permeability of living cells can be drawn from his experiments.

I have observed also the stainability of the yolk platelets of the frog's egg in dilute solution of analine dyes in the acid and alkaline condition. These bodies are likewise more readily stainable by the free color acids and bases than their salts. I will discuss this matter later.

The acid solutions contained $\frac{N}{1000}$ HCl; the alkaline $\frac{N}{1000}$ NaOH in tap water; neutral solutions were of glass distilled water. The acid had no effect on the Spirogyra during the short time of the experiment, under four hours. The dyes (all prepared by Grübler and Co.) were of such concentration as to give a very light colored solution in a layer 3 cm. thick. Many dyes cannot be satisfactorily studied because they become colorless very rapidly in alkaline solution and less rapidly in neutral solution. The letter B in the next table placed after the dye signifies that it is basic; A signifies an acid dye. In the last column is given the stainable power of the yolk platelets of the frog's egg in the same dilute dye solutions used in the study of Spirogyra. The platelets were obtained from the ovarian eggs of Rana catesbiana. They are regarded by McClendon ('10) as a lecithalbumin "composed of 6 per cent lecithin and 94 per cent batrachiolin, a nucleo-albumin containing 1.2 per cent phosphorus, 1.3 per cent of sulphur, and 15 per cent of nitrogen."

The entrance of all the basic dyes studied is indicated in Spirogyra by precipitation with tannic acid as fine colored granules, just as is neutral red. On the contrary the penetration of the acid dyes is accompanied by a combination of the dye with the cell proteids, nucleus and pyrenoids appearing colored first, then cytoplasm and chlorophyll bands. The spiral bands are often distorted, the nucleus swollen and turgor is invariably lost. This is true of all the acid dyes given in the following table and is indicated by the word stained. As mentioned above the appearance of cell staining is often given by a union of the dye with the cellulose wall but this is readily detected by high magnification. It is obvious that only the presence of colored granules in the sap vacuole or the staining of the protoplasm should be regarded as criteria of permeability.

2. Relation to Overton's lipoid theory

It will be seen from table 1 that it is the free color bases or acids which enter cells and not their salts and this is the same result which Robertson obtained in studying the solubility of dyes in

TABLE 1

DYE	REACTION	COLOR	SPIROGYRA	YOLK PLATELETS
Neutral red (B)	acid	pink	colorless	colorless
	neutral	pink	red granules	colorless
	alkaline	yellow	red granules	colorless
Methylene blue (B)	acid	blue	colorless	colorless
	neutral	blue	blue granules	very faint blue
	alkaline	blue	blue granules	blue
Saffranin (B)	acid	red	colorless	colorless
	neutral	red	red granules	very faint red
	alkaline	red	red granules	red
Methyl violet (B)	acid	violet	colorless	colorless
	neutral	violet	violet granules	faint violet
	alkaline	violet	violet granules	violet
Bismark brown (B)	acid	orange yellow	colorless	colorless
	neutral	orange yellow	red brown granules	faint yellow
	alkaline	lemon yellow	red brown granules	faint yellow
Thionin (B)	acid	blue	colorless	colorless
	neutral	blue	blue granules	faint blue
	alkaline	light purple blue	blue granules	blue
Chrysoidin (B)	acid	orange yellow	colorless	colorless
	neutral	orange yellow	brown granules	faint yellow
	alkaline	lemon yellow	brown granules	faint yellow
Tolyindin blue (B)	acid	blue	colorless	colorless
	neutral	blue	blue granules	very faint blue
	alkaline	blue	blue granules	blue
Eosin (A)	acid	vermillion	stained red	red
	neutral	vermillion	colorless	red
	alkaline	vermillion	colorless	faint pink
Bordeaux red (A)	acid	pink	stained pink	red
	neutral	pink	colorless	red
	alkaline	pink	colorless	colorless
Saüreviolet (A)	acid.	blue violet	stained blue	violet
	neutral	blue violet (fades slowly)	unstained	violet
	alkaline	blue violet (fades slowly)	unstained	colorless
Aurantia (A)	acid	light yellow	stained light yellow	yellow
	neutral	yellow	unstained	yellow
	alkaline	yellow	unstained	faint yellow

fatty substances and fat solvents. It must not be forgotten that, as Mathews ('98) showed, the basic stains yield colored precipitates with proteids only in alkaline solution, the acid only in acid solutions. The same was found to be true of the staining of coagulated proteids as egg albumen. We should expect therefore that the lecithalbumen platelets of the frog's egg would show the same staining relations that Mathews found for coagulated albumen, even from very dilute solutions. It is probable that the acid or alkali affects the lecithalbumen as well as the dye.

We might draw the conclusion from this that a dye only enters a cell when it combines with the surface membrane.[4] Yet I have never noticed that the plasma membrane of any cell becomes stained in dilute solutions of basic dyes. The most conspicuous fact connected with the staining of plant cells is that the stain passes through the cell protoplasm without affecting it in the least and collects in the vacuole.

My studies on dyes have not been extensive enough to warrant generalizations as to the classes of dyestuffs for which cells are permeable nor as to the nature of the cell surface. It appears to be true—as a general rule, to which there are exceptions—that the substances (including alkaloids, alkalies, dyes, anaesthetics, etc.) more soluble in fat solvents or fatty substances than in water, penetrate cells with practically no resistance, while those compounds insoluble in ether and fats meet a marked resistance at the cell boundary as Overton has postulated. But whether we are to conclude from this that the boundary is lipoid in nature is quite another question. The evidence on this point is far from conclusive. Indeed, Traube has shown that the lipoid soluble substances, the easily permeating substances, are those having the greatest tendency to lower the surface tension of water in air, and, according to his theory of osmosis to pass into the phase of greater surface tension (into the cell). No lipoid membrane separating the two phases is required.

[4] Mathews ('10) has recently concluded that the dyes penetrate by "combination with substances in the peripheral layer such as lecithin and the electro-negative proteins, soaps and possible other substances." (p. 218). He regards the taking up of basic dyes by lipoid solvents, which act as weak acids, as a chemical combination.

3. Mechanism of staining animal cells

Animal cells show the same relations toward neutral red as do
plant cells: Paramoecia were placed in the following solutions:

A.   10 cc. tap water + 0.1 cc. $\frac{N}{10}$ HCl $(\frac{N}{1000})$ + 1 drop 0.02 % neutral red.
B.   10 cc. tap water + 0.1 cc. $\frac{N}{10}$ NaOH $(\frac{N}{1000})$ + 1 drop 0.02 % neutral red.
C.   10 cc. tap water + 1 drop 0.02 % neutral red.

After 30 minutes the individuals in A are unstained while those
in B and C are very deeply stained.   In one hour the Paramoecia
in A show a faint pink color in some of their vacuoles, and later
they become noticeably stained.   This is probably due to the
fact that the animals are constantly forming new food vacuoles.
A small amount of neutral red enters with the fluid of the vacuole.
The acid passing in at the same time is neutralized and the dye
may then pass the wall of the vacuole and stain certain granular
bodies in the protoplasm.   Paramoecia stain after some time in
acid solutions not because the dye may pass the surface mem-
brane but because it is engulfed along with the food of the organ-
ism.   The food eaten may itself be stained.

If paramoecia stained in neutral red are centrifuged in an elec-
trical centrifuge for one and one half hours it is easy to dif-
ferentiate the bodies with which the dye unites.   Six more or less
distinct zones may be distinguished.   These very soon mix again
due to the constant rotation of the protoplasm.   Their relative
volumes are indicated in fig. 1.

Only two substances in Paramoecium are found stained (1)
the food and granules in some of the vacuoles; (2) the minute
granules which often form a ring about the food vacuoles.   Macro
and micronucleus, trichocysts, cilia and the clear fluid portion of
the protoplasm of the living organisms are quite unstained.

The staining of marine eggs is essentially similar to the stain-
ing of Paramoecium.   Owing to the presence of bicarbonates and
phosphates in sea water considerably more acid must be added,
than is necessary to change the color of neutral red from yellow
to red, before all the dye is actually in the acid condition and
consequently, before the dye will fail to enter the eggs.   About
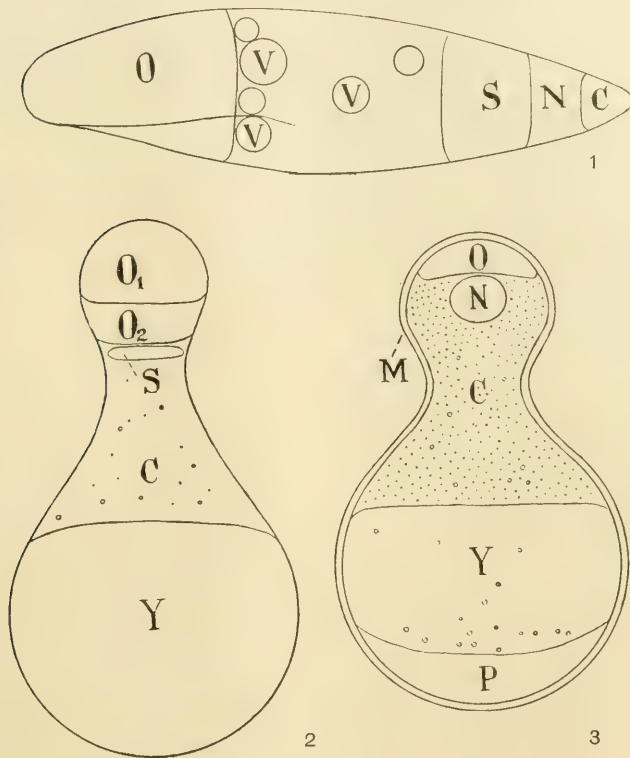0.5 cc. $\frac{N}{10}$ HCl to 100 cc. sea water is sufficient to bring about

Fig. 1   Diagram of the areas which may be distinguished in a centrifuged Para-
moecium, (electric centrifuge 1.5 hours at a radius of 6 cm.) stained in neutral
red.

O, oil (?) globules; V, food vacuoles, the contained matter often red stained;
S, granules stained in neutral red; N, macronucleus; C, crystals.   If the organism
is uninjured, there is a very rapid redistribution of substances in the protoplasmic
circulation.

Figs. 2 and 3   Diagrams of the distribution of substances in centrifuged Chae-
topterus (2) and Arbacia (3) eggs stained in neutral red.   Elongation in the axis
of the force has taken place.   O, O_1, O_2, oil globules; in Chaetopterus of two
sizes and of two densities O_1 and O_2; N, nucleus; C, clear area; in Chaetopterus
containing a few scattered yolk and minute red stained granules; in Arbacia
containing at the surface a few pigment bodies (chro matophores) and numerous
minute stained granules, quite unmoved by the cent rifuge.   At the time of fer-
tilization these disappear, apparently going to form the substance which passes
out of the egg and hardens to a fertilization membrane.   Y, yolk; in Chaetopterus
appearing pink from numerous minute red stained granules most of which col-
lect in a mass (S) just under the oil.   P, the pigment granules, appearing dark
red from absorption of the neutral red dye.

the color change of neutral red. The yellow color returns to some extent on standing. Both Arbacia and Asterias eggs stain in 100 cc. sea water $+$ 2 cc. $\frac{N}{10}$ HCl but fail to stain if 3 cc. $\frac{N}{10}$ HCl is added, even after one hour. They are quite transparent and uncoagulated. About half the egg of Asterias coagulate in 100 cc. sea water $+$ 5 cc. $\frac{N}{10}$ HCl after one hour's time and those which are opaque and coagulated become faint pink in color. Apparently the dye is adsorbed by the proteid coagulum.

The mechanism of absorption of neutral red is practically the same as in Paramoecium. In all the eggs thus far studied (Cumingia, Arbacia, Asterias, Toxopneustes, Hipponoë, Holothuria and Chaetopterus) it combines with very definite granules which often differ in color, always in specific gravity and generally in size, from other granules in the egg. In markedly pigmented eggs like Arbacia or Cumingia, it is the pigment granules which become stained, but in the eggs of Holothuria, the large yolk granules are orange and the dye absorbing granules are small and colorless. The latter are much the heaviest granules present and pass to the outer pole of the egg when centrifuged. That this is not due to an increase in weight from taking up of the dye may be shown by first centrifuging the eggs and then staining them. Exactly the same areas stain as if the experiment had been reversed, the eggs first stained and then centrifuged.

The two statements made above are true of all the eggs studied except Chaetopterus. The stainable granules of Chaetopterus are specifically different from other granules in the egg, but they are not the heaviest. The difference is best made clear by reference to figs. 2 and 3.

The red area is found to be just under the oil and to consist of globules of varying size which have apparently been formed by fusion of very minute red granules. These may be easily seen over the clear area and scattered throughout the yolk mass, giving a pinkish tinge to that region.

A similar aggregation of minute granules to form larger granules occurs in Toxopneustes also. Both fertilized and unfertilized eggs at first stain 'diffusely,' i.e., the dye is localized in very minute granules, visible under high magnification. More and more of

these minute granules take the dye and at the same time fuse together to form clearly defined red bodies much like the chromaphores of Arbacia eggs. The final stage is more rapidly attained in eggs with fertilization or artificial membranes. Inasmuch as the conditions under which the dye unites with the granules in the two types of eggs, fertilized and unfertilized, may be different, we cannot at present draw conclusions as to differences in permeability to dyes in the two types of eggs.

In brief the mechanism of staining Paramoecium or marine eggs is as follows: The dye enters the egg as the weak base, yellow in color, and combines with a specific insoluble substance present, in the form of granules. The combination resulting, likewise insoluble and comparable to a salt, is red in color just as are the water soluble salts of neutral red. The exact chemical nature of the granules with which neutral red combines is unknown.

THE PENETRATION OF ALKALIES

1. Method and previous results

Of the many methods which may be used in studying the penetration of various substances, the color change of an indicator within the cell is the simplest and most delicate for the detection of acids and alkalies. Many plant cells contain natural pigments which may serve as indicators. Both Pfeffer and DeVries made use of such pigments in their studies on permeability.

DeVries ('71, p. 24) noted that the red sap of beet cells becomes brown to yellow brown in dilute NH_4OH and the red color comes back again on washing in pure water.

Pfeffer ('77, p. 140) showed that the red sap of Pulmonaria petals and of the stamen hairs of Tradescantia becomes first blue then greenish in dilute ammonia. KOH and K_2CO_3 act like ammonia (p. 141). Pfeffer regards the dead and the living plasma to be similarly easily permeable for dilute alkalies as well as acids. This is the view stated in botanical text-books at the present day and the subject is dismissed with few words and without further

comment. Evidence will be given in the body of this paper to show that the generally accepted view is only in part the truth, so far at least as the penetration of alkalies is concerned.

Animal cells suitable for experimental studies contain no pigment exhibiting a marked color change in alkalies. We may overcome the diffiulty by introducing a dye which does so change. Neutral red is excellent for the purpose. In solution, it turns from red to yellow in an H ion concentration of 1.10^{-7} to 1.10^{-8}, and is perfectly harmless for the cells providing they are not allowed to take up over a certain maximal amount. For the sake of comparison neutral red was employed in plant cells as well.

I have endeavored to answer for the alkalies the following general questions, many of which have already been settled for other classes of substances by previous workers in the field of cell permeability.

1. Do plant and animal cells exhibit essentially similar permeability relations for alkalies?

2. Do living and dead cells exhibit similar permeability relations for alkalies?

3. Are there distinct classes of alkalies in regard to permeability?

4. Are the effects of different alkalies reversible or irreversible after an equal amount (OH ion concentration) has entered the cell?

5. May the composition of the medium affect permeability without irreversibly injuring the cells themselves?

6. Are there any relations between functional changes and permeability for alkalies?

7. May alkalies produce marked functional changes without entering the cell?

Many of these questions can be decided only with an especially favorable type of cell and the evidence one way or the other has therefore been given in describing the permeability phenomena of that particular cell. The connection between the penetrating power and the physical properties of the various alkalies are taken up in the discussion. I have taken the greatest precaution to

exclude sources of error and make sure that the permeability of *living* cells is in reality the problem studied, a point not always carefully guarded against. For this reason the observations and experiments which follow are recorded in considerable detail.

2. Objections to the use of an indicator within the cell

Despite the fact that indicators offer the most delicate means of detecting alkalies (and neutral red is exceedingly sensitive in this respect, reacting to Na_2HPO_4, there are four possible complications arising from its use in the cell, all of which would tend to make the amount of alkali entering, appear less than that which actually entered. Or, to put it in another way, more alkali would enter than we should calculate, judging from the concentration at which the color change takes place in pure water.

1. In determining alkalinity in a test tube we have only a very weak solution of the indicator: in the leaf cells of Elodea, neutral red is in concentrated solution (otherwise it would be quite invisible in so narrow a space) and also in combination with some complex organic acid. Enough alkali must enter to convert it all into the free base (ROH) before the color change occurs.

2. In Spirogyra neutral red is accumulated as a tannin compound which becomes straw color in the presence of alkali and red again on adding acid and begins to dissolve. This compound as well as the combination formed by neutral red with the granules of animal cells require a greater concentration of alkali or a longer time, to turn them yellow than does the pure dye in distilled water. By treating cells with chloroform or by rupturing their surface, as described under each organism investigated, it is possible to exclude the complication due to the above mentioned conditions. The concentration of alkali may be found which brings about a color change instantaneously.

3. The most serious objection to the use of neutral red as an indicator within the cell is, as I have determined experimentally, that in the presence of proteids like egg albumen, the dye (in solution) is unaffected by a certain amount of added alkali which

apparently enters into combination with the albumen. The following experiment illustrates this:

A stock solution of unknown strength in 0.1 N NaCl was made from Merck's powdered egg albumen. When filtered it was opalescent.

To 5 cc. was added one drop of 0.05 per cent neutral red and the solution titrated with $\frac{N}{50}$ KOH. 0.6 cc. was added before the original pink color began to turn and 0.9 cc. $\frac{N}{50}$ KOH before it was yellow. Phenolpthalein was then added and the solution found to be neutral. In all 1.6 cc. was added before the phenolpthalein began to turn pink and 2.0 cc. before it became distinctly pink.

0.6 cc. $\frac{N}{50}$ NH$_4$OH was also required before the neutral red began to fade and 1.1 cc. before a distinct yellow appeared. The solution was still neutral to phenolpthalein and 3.00 cc. were required before a light pink appeared.

The effect of chloroform on the power of neutralization was also determined. The solutions compared were:

A   5 cc. albumen + 10 cc. water + 1 drop 0.1 per cent neutral red.
B   5 cc. albumen + 10 cc. chloroform-saturated water + 1 drop 0.1 per cent neutral red.

Exactly the same amounts of $\frac{N}{50}$ KOH and $\frac{N}{30}$ NH$_4$OH were required to induce the color change in A as in B. Chloroform has no effect on the combining power.

Yet one drop (0.05 cc.) of $\frac{N}{50}$ KOH or NH$_4$OH is more than sufficient to render 0.1 N NaCl solution alkaline to both neutral red and phenolpthalein.

Any difference in permeability between KOH and NH$_4$OH—and there is a very great difference—cannot therefore be attributed to a difference in combining power of K and NH$_4$. The use of chloroform in killing cells must affect the surface layer of the cell in some way, and not the combining power of the cell proteids within.

4. It is quite possible that the cell (especially plant cells) may actively secrete an acid which neutralizes the alkali as it enters. But when we consider that the mass of the material studied relative to that of the alkaline solution is very small and that the

KOH, let us say, would continue to diffuse in so long as neutralized at a practically constant rate, it would require an enormous production of acid on the part of the cell to take care of the KOH entering. For this reason alone it seems safer to consider that the inorganic alkalies do not enter (without affecting the surface membrane) rather than that they are neutralized as they enter.

There is, indeed, some evidence that an acid is secreted into the vacuole of Elodea cells. If red stained leaves are placed in $\frac{N}{40}$ NH_4OH until color change occurs and then are immediately transferred to pure water, the cells are uninjured and the red color returns. It is often a distinctly brighter red than before. I have never noticed that Elodea cells placed in solutions of inorganic alkalies became a brighter red before the color change finally occurred.

Again red stained leaves treated for one minute with chloroform water appear a slightly brighter red than the control. The chloroform apparently induces the formation of an acid which collects in the vacuole. Yet such leaves placed in $\frac{N}{40}$ NH_4OH are decolorized slightly more rapidly than normal leaves and in $\frac{N}{40}$ KOH, 30–40 times more rapidly. The experiment shows the negligible effect of the acid in neutralizing entering alkali.

The above considerations make the calculation of the strength of any alkali entering a cell very difficult. We can state this much, however, we can give the concentration of alkali in terms of the color change of the neutral red combination in each particular cell, and the relation of this color change to structural or functional changes brought about by the presence of the alkali. We must assume that when red becomes yellow the OH ion concentration of the various alkalies is equivalent.

It must be borne in mind that the indicator in both Spirogyra and Elodea is in the cell vacuole and that the alkali to reach it must pass through the cell (plasma) membrane, the protoplasm, and the vacuolar membrane. On the contrary, the indicator in animal cells is in the protoplasm.

3. Experiments with plant cells

a. *Strongly dissociated alkalies:* The leaf of Elodea, except at the midrib, is only two cells thick, a layer of small cells constituting the lower surface, a layer of much larger cells above. There is great variation in different leaves in the time for the color change to take place, as well as individual differences among the separate cells. A consequence of the latter fact is the decolorization (in K, Na, or $N(C_2H_5)_4OH$) of the leaf in patches, groups of red cells becoming yellow before others. The phenomenon is not marked in Ca, Sr, and Ba. Spirogyra exhibits the same variability as Elodea.

A comparison between the inorganic hydroxides was made with $\frac{N}{10}$ alkali in tightly corked bottles, to prevent absorption of CO_2. The water was redistilled from glass and was non-toxic to Spirogyra, Elodea and Paramoecium. Paramoecium is especially sensitive to commercial distilled water and may be used as an indicator of the purity of a water. Elodea showed rotation in commercial distilled water after 24 hours. Nevertheless the rate of entrance of NaOH is more rapid when dissolved in commercial distilled water than in pure redistilled water and more rapid in pure redistilled water than in tap water.

The following table constructed from many experiments gives the relative rate of penetration of $N(C_2H_5)_4OH$, Na, K, Ca, Sr, and Ba hydroxides[5]. The actual times varied somewhat in individual experiments, but the relation order of penetration was

Penetration of $\frac{N}{10}$ alkali into Elodea leaves.

	MINUTES		MINUTES
NaOH	25	$N(C_2H_5)_4OH$	30
KOH	22	NH_4OH	0.5
$Ca(OH)_2$	23	Methyl amine	1
$Sr(OH)_2$	15	Trimethyl amine	2
$Ba(OH)_2$	15		

[5] I am indebted to the Chemistry Department of Columbia University for methyl, dimethyl, trimethyl, ethyl, propyl, and isopropyl amines. Tetraethyl-ammonium hydroxide was obtained from Merck and Co. The inorganic hydroxides were Eimer and Amend's C. P. with the exception of $Ba(OH)_2$, which was manufactured by Kahlbaum.

fairly constant. In all the experiments on alkalies, N means normal, equivalent to corresponding normal solutions of HCL.

The rate of entrance when the cells have been killed in various ways is shown in tables 2 and 3.

TABLE 2

Times for different concentrations of NaOH *to decolorize the neutral red tannin compound within Spirogyra threads. In column A are figures for the living plant; in B for threads killed by boiling water and stained in neutral red; in C for threads killed by* $\frac{N}{10}$ HCL *(3 mins.) and washed free of acid; in D killed by* $CHCl_3$ *water (1 min.), and in E the threads were killed by a saturated solution of* $HgCl_2$ *(3 mins.) and washed in water. The* NaOH *was dissolved in tap water and the solutions tested in corked bottles.*

CONCEN-TRATION OF NaOH	A	B	C	D	E
$\frac{N}{10}$	0.5 minutes	instantly	instantly	instantly	
$\frac{N}{20}$	2.5 minutes	instantly	instantly	instantly	
$\frac{N}{30}$	9 minutes	instantly	instantly	instantly	
$\frac{N}{40}$	40 minutes	instantly	instantly	< 0.5 minutes	< 0.5 minutes
$\frac{N}{50}$	70 minutes	instantly	instantly	< 0.5 minutes	< 0.5 minutes
$\frac{N}{60}$	1.5 hours	instantly	instantly	< 0.5 minutes	< 0.5 minutes
$\frac{N}{70}$	3 hours	instantly	instantly	< 0.5 minutes	< 0.5 minutes
$\frac{N}{80}$	2 hours	instantly	instantly	< 0.5 minutes	< 0.5 minutes
$\frac{N}{160}$	<18 hours	instantly	0.5 minute	1.0 minute	2 minutes
$\frac{N}{320}$	>18 hours	instantly	1 minute	3 minutes	10 minutes
$\frac{N}{640}$	>18 hours	1 minute	2 minutes	8 minutes	
$\frac{N}{1280}$		2 minutes		18 minutes	

TABLE 3

Penetration of different concentrations of NaOH *into Elodea leaves. The* NaOH *was dissolved in tap water.*

CONCENTRATION OF NaOH	NORMAL LEAVES	LEAVES KILLED BY CHLOROFORM	LEAVES KILLED BY BOILING WATER, AND DEEPLY STAINED IN NEUTRAL RED
$\frac{N}{10}$	2 minutes	instantly	instantly
$\frac{N}{20}$	9 minutes	1 minute	instantly
$\frac{N}{40}$	45 minutes	2 minutes	instantly
$\frac{N}{80}$	3 hours	5 minutes	instantly
$\frac{N}{160}$	unaffected after 18 hours	8 minutes	2 minutes

It is found that all alkalies may enter with practically no resistance providing the cell surface has been destroyed in some way; it is immaterial how. Thus in $\frac{N}{80}$ NaOH Spirogyra threads retain their red color about 3.5 hours, but if treated with $CHCl_3$

saturated water and then placed in $\frac{N}{80}$ NaOH the red precipitate becomes yellow immediately. On adding dilute HCl the thread again becomes red and the precipitate begins to dissolve.

As shown above, chloroform has no effect on the alkali combining power of egg albumen and presumably none on cell proteids. It must be that the inability of the alkali to enter is due rather to the inability to pass the surface layer of the Spirogyra cell than to a neutralization by proteid or acid after passing this layer.

The color change in the tannin precipitate takes place practically instantaneously in $\frac{N}{640}$ NaOH (or KOH, Ca(OH)$_2$, Ba-(OH)$_2$ or Sr(OH)$_2$), if we cut the cell transversely so as to allow ready mixture of solution and precipitate. In weaker concentrations the color change also occurs but it takes a much longer time. Thus, dead Spirogyra filamants (red stained) become decolorized in less than 15 hours in $\frac{N}{5120}$ NaOH, while the control in water remained red.

Every cell which I have tested (Cabomba rosafolia, containing a natural red pigment, Elodea Canadensis, Spirogyra, Paramoecium, Vorticella and various marine eggs) has proved to be resistant to the entrance of the inorganic hydroxides, a condition which is lost on death (by chloroform, HCl, heat coagulation, drying, etc.) This post-mortem increase of permeability has been so often emphasized by many writers for very diverse substances that it hardly requires special confirmation for the alkalies, except as showing the degree of impermeability which the normal living cell possesses.

b. *Weakly dissociated alkalies:*—Exactly opposite results are obtained when ammonia and its primary, secondary and tertiary alkyl substitution products[5] are studied, instead of the inorganic hydroxides. All these substances pass into the cell with very little if any resistance. Elodea is more suited to experimentation than Spirogyra[6] because the color change is more marked (tables 4 and 5).

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[6] If a red stained filament of Spirogyra is placed $\frac{N}{40}$ NH$_4$OH it becomes dark green in one minute. On washing in water the red color soon returns. In weaker solutions of ammonia the formation of the NH$_4$—tannate compound prevents a sharp color change in the neutral red-tannate compound.

TABLE 4

Effect of NH$_4$OH *on red Elodea leaves. Glass distilled water used. Solutions in corked bottles*

CONCENTRATION OF NH$_4$OH	$\frac{N}{10}$	$\frac{N}{20}$	$\frac{N}{40}$	$\frac{N}{80}$	$\frac{N}{160}$	$\frac{N}{320}$	$\frac{N}{640}$	$\frac{N}{1280}$
Normal leaf..	instantly	1 minute	1 minute	1½ minute	3 minutes	6 minutes	Not entirely yellow after 30 minutes	Unaffected after 1 hour. Slightly affected after 18 hours
Chloroform treated leaf.	instantly	instantly	¾ minute	1 minute	3 minutes	6 minutes	Dye diffuses out of leaf	

In Spirogyra, entrance of ammonia may be indicated also by the precipitation of tannin, and this method has been used by Overton ('97) who found that ammonia, the primary and secondary amines penetrate readily, the tertiary amines and quaternary ammonium bases do not, behaving in this respect like inorganic hydroxides. So far as I have observed trimethyl amine enters cells readily but not so quickly as the methyl or dimethyl derivitives of ammonia.

If an Elodea leaf has remained in a solution of $\frac{N}{40}$ NaOH (or K, Ca, Sr, Ba, N(C$_2$H$_5$)$_4$ hydroxides of any concentration) until the color change to yellow has occurred, and then is immediately placed in pure water it is found that the leaf has been killed and the red color never returns.[7] The capacity of again accumulating dye is likewise lost. The entrance of a sufficient quantity of inorganic alkali to affect the neutral red produces irreversible changes in the cells themselves.

But if a similar Elodea leaf is placed in an $\frac{N}{40}$ NH$_4$OH solution the color change occurs almost instantly (about one minute). Furthermore, on transferring to pure water, the red color in the cells and the protoplasmic rotation characteristic of the plant returns. Evidently death of the leaf does not necessarily ensue from the

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[7] Except on immediately adding an acid. This shows that the dye is actually changed to yellow and not reduced to a colorless substance. The actual yellow color may be obscured to some extent by the green chlorophyll present.

entrance of a concentration of OH ions sufficient to affect the neutral red combination. In the case of the inorganic alkalies and $N(C_2H_5)_4OH$ death is to be referred to two possible causes.

First, and of greatest importance, the alkali only enters after the cell surface has been destroyed. This is best illustrated by comparison of the mode of entrance of NH_4OH and $NaOH$ into Paramoecium (p. 546) and is strong evidence in support of Hoeber's ('06, pp. 260, 266–267) theory that the strong electrolytes, in general, produce their effects by a change in the colloids of the cell surface and not of the cell interior.

Second, the combination of the strong alkalies with the cell surface proteids may be irreversible, whereas the combination of the weak ammonia is easily reversible.

The cell surface of the living as compared with the dead (by chloroform treatment) cells offers a highly resistant barrier to the entrance of the strong alkalies but both living and dead cells are almost equally permeable for the weak alkalies (see table 4). This suggests, but does not prove, that if small quantities of $NaOH$ could enter without affecting the membrane, the cell would be as unharmed as in NH_4OH.

Ammonia has likewise a toxic effect but it is only manifest after a longer exposure, and is quite independent of the entrance of ammonia into the cell. Red Elodea leaves recover if placed in fresh water immediately after decolorization in $\frac{N}{10}$ NH_4OH. If left for five minutes the dye becomes red again but the cells eventually die. If left over 30 minutes even the red color fails to return. The leaf is of course killed.

The inorganic hydroxides (and $N(C_2H_5)_4OH$) only enter the cell after they have affected the normal impermeability—in other words after they have rendered its surface permeable to themselves. It seems best, then, to speak of a resistance of the cells for the strong and a permeability of the cell for the weak alkalies.

Reversibility of the neutral red color change is quite independent of the death of the cell. The red returns after decolorization by the amines although they produce fatal after-effects.

It likewise returns in cells first decolorized in ammonia and then killed with chloroform water, and more rapidly than in the

control.   Chloroform water induces the formation of an acid in
the vacuole and the production of acid in solution of weak alkalies
is probably one factor in the return of the red color.

Failure of Elodea leaves to become red when once turned yellow
by KOH is not due to the longer time required by the KOH to
bring about the color change (the neutral red diffusing out slowly
in the interval), because the leaves become yellow within one
minute, if placed in $\frac{N}{10}$ KOH yet in pure water the red does not
return.

We must seek an explanation of the reversibility of the NH_4OH
change, the irreversibility of the KOH change in the different
degrees of hydrolytic splitting of the respective NH_4 and K salts
of the acid with which the neutral red combines.   If R is the acid
radical within the plant and D the neutral red radicle, the reactions
may be represented thus:

$$RCOOH + DOH = RCOOD + H_2O.$$
$$RCOOD + NaOH = RCOONa + DOH$$
$$RCOOD + NH_4OH = RCOONH_4 + DOH$$
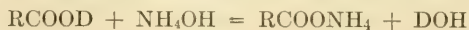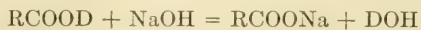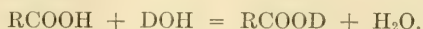
The $RCOONH_4$ salt of a weak base and a weak acid undergoes
a greater hydrolytic splitting than the $RCOONa$ combination, the
NH_4OH formed diffuses rapidly away and recombination of
$RCOOH$ and DOH again take place.

The difference in resistance of the plasma membrane to NH_4OH
and $NaOH$ is strikingly shown in the following experiment.   If
we remove Elodea leaves, after decolorization in NH_4OH to
chloroform water (one minute) and then place them in $\frac{N}{50}$ $NaOH$
there is never a return of the red color.   Chloroform destroys the
plasma membrane and the KOH may penetrate rapidly.   On
the other hand, if the leaf decolorized in $\frac{N}{40}$ NH_4OH is placed in
$\frac{N}{50}$ $NaOH$, without previous chloroform treatment, its cells become
red again just as they would in pure water.   The conditions for
demonstrating any entrance of $NaOH$ are here most favorable,
the neutral red is already in the alkaline condition, the proteids
have taken up the amount of NH_4OH necessary before and alka-
line reaction may be indicated, yet the NH_4OH *diffuses rapidly*

outward while the NaOH cannot pass in to maintain the dye in the yellow condition. Eventually of course the leaf in $\frac{N}{50}$ KOH becomes yellow just as does the control.

The amines show a behavior similar to NH_4OH but, with the exception of trimethyl amine, are considerably more toxic and produce after effects which lead to the death of the cell. The entrance of just enough to affect the neutral red is typically fatal (table 5).

TABLE 5

Effect of NH_4OH and amines on red Elodea leaves. Concentration, $\frac{N}{40}$ in glass distilled water. The red color disappears (decolorized) in less than 2 minutes in all solutions. The leaves are then transferred to (A) tap water; (B) $\frac{N}{100}$ NaOH. Columns A and B give the results, respectively.

$\frac{N}{40}$	A	B
NH_4OH..........................	Cells become red in 15–30 minutes and still alive after 18 hrs.	Cells become red again in 15–30 mins. and only turn yellow when control turns yellow
NH_3CH_3OH......................	Cells become red in 15–30 minutes but dye diffuse out. Colorless after 18 hours.	Cells remain colorless
$NH_2(CH_3)_2OH$	Cells become red·in 15–30 minutes but dye diffuse out. Colorless after 18 hours.	Cells remain colorless
$NH(CH_3)_3OH$....................	Same as NH_4OH	Same as NH_4OH
$NH_3(C_2H_5)OH$..................	Same as NH_3CH_3OH	Same as NH_3CH_3OH
$NH_4(C_2H_5CH_2)OH$.............. (normal propyl amine)	Same as NH_3CH_3OH	Same as NH_3CH_3OH
$NH_3(CH_3)_2CHOH$ (isopropyl-amine)..........:.............	Same as NH_3CH_3OH	Same as NH_3CH_3OH
Control..........................	Red and normal after 18 hours	Red > 2 hours

Leaves treated with $CHCl_3$, after decolorization in any of the above solutions, and placed in $\frac{N}{10}$ NaOH never become red again; if placed in tap water all tend to become red but the red dye eventually diffuses out of the cells because the cell surface has been affected by the chloroform.

c. *Permeability of cells exhibiting protoplasmic rotation.* There appears to be no marked difference in the resistance of 'rotating' and quiescent cells to the penetration of NaOH or KOH. In a number of experiments in which individual cells were watched rotating cells became yellow before non-rotating or *vice versa.* Comparisons of whole leaves are not of much value because of their great variability in resistance to NaOH.

Judging from Hörmann's ('98) researches and the comparison he has drawn between the rotating plant protoplast and muscular contraction a difference in permeability was to be looked for. The cessation of streaming induced by thermal, mechanical, chemical and electrical means strongly suggests that a shock-stoppage is comparable to muscle contraction and depends on a similar conditioning change. Even the details connected with electrical stimulation run parallel. According to Hörmann the passing of a constant current through a rotating Nitella cell causes a cessation of movement at the cathode on the make and at the anode on the break. While the current is passing the streaming is slower at the cathode. A wave of shock stoppage may be propagated from one region of a leaf to another and when tested electrically the stopped regions are found to be negative to rotating ones just as the region of a muscle in contraction is negative to an uncontracted area.

We might therefore consider that protoplasmic streaming is in some way connected with a high degree of electrical polarization, a low surface tension and a surface membrane relatively impermeable to soluble substances, states typical of the resting condition of many types of cells. Each of these three conditions undergoes a change in the opposite direction, on 'stimulation' of the cell.

As stated before I have been unable to detect any constant differences in permeability (or resistance) of rotating and non-rotating cells to KOH or NaOH. It is quite possible that several factors each of which alone may be sufficient to prevent rotation, are involved.

d. *The concentration of alkali which stops rotation:*—In NH_4OH ($\frac{N}{10}$ to $\frac{N}{80}$) rotation ceases just at the point where the bright pink sap begins to turn dull pink before finally becoming yellow. In NaOH the rotation ceases, begins again and finally ceases permanently long before the initial dull pink of the color change appears. The description of the two experiments in which the changes in individual cells was observed is as follows:

Ammonia: Red stained Elodea leaves are placed in $\frac{N}{20}$ NH$_4$OH. Cells become yellow in less than one minute. Rotation ceases at the time the color change begins ($\frac{3}{4}$ min.). After 1 min. the alkali is replaced by water. The bright red color begins to return (20–25 mins.) and the cells are as red as they were originally in 1 hour. In 1 hour, 10 mins. jerky rotation begins and in 2 hours the original rapid rotation may be observed.

Sodium hydrate: Red stained Elodea leaves are placed in $\frac{N}{40}$ NaOH under the microscope. Rotation ceases in about one minute, begins again slowly in 5–10 minutes, stops again after 15 minutes longer, and the red sap only begins to turn dull pink to yellow after 45 minutes to 1 hour. If the alkali is replaced by water, the sap never becomes red again and rotation never returns.

e. The effect of added substances on the penetration of NaOH and KOH. As indicated in table 4 even chloroform treatment hardly increases the rate with which NH$_4$OH may enter Elodea cells. Ammonia enters living cells as rapidly as dead ones. NaOH enters dead cells nearly as rapidly as ammonia, but living cells offer a high resistance to its passage. This resistance may be decreased by the addition of chloroform to the medium in amounts too small to have any irreversible effects in the absence of NaOH. On comparing the time for NaOH or KOH to decolorize red stained Elodea leaves in $\frac{N}{40}$ solution alone and in $\frac{N}{40}$ solution plus dilute chloroform, alcohol, urea, glycerine, and various salts it is found that all have the effect of shortening the time which it takes for the NaOH to enter. The analysis of the experiment is somewhat complex. The effect of the added substance may be on the plasma membrane or on the alkali (affecting its dissociation or combining to form more toxic compounds); or the alkali may allow the more ready entrance of the added substance with consequent rapid destructive action and death of the cell which leads also to easy penetration of alkali. In other words, the action of two substances together may be additive.

On account of the complexity and difficulty of interpreting results I discontinued further experimentation along this line. A few experiments are given. The effect of dilute chloroform solution must be attributed to decrease in resistance of the cell

surface. Such a concentration would have no effect on the alkali or vice versa.

If the urea glycerine or salts change the condition of the plasma membrane it is surprising how little effect this has on the proto-plasmic streaming, which continues for many hours in solutions of these substances.

If leaves are selected from the same or neighboring whorls on the same plant concordant results may be obtained. But different plants and young and old leaves exhibit the greatest variations in resistance to NaOH, as separate experiments will show. The solutions were contained in tightly corked glass vials to prevent absorption of CO_2. Two to four leaves were tested in each experiment.

Experiment 1. A. $\frac{N}{40}$ NaOH, $\frac{1}{6}$ saturated with $CHCl_3$ tap water—decolorized in 13 minutes.

B. $\frac{N}{40}$ NaOH in tap water—decolorized in 90 minutes. Rotation ceases in leaves in this solution in $<$ 15 minutes, but if removed to tap water (after 15 minutes) begins again in 16–20 minutes.

C. $\frac{1}{6}$ saturated $CHCl_3$ in tap water—Rotation ceases, but if removed to tap water (after 15 minutes,) begins again in $<$ 15 minutes.

One-sixth saturated chloroform increases the permeability of Elodea cells to urea. Leaves removed from solution C after ten minutes plasmolyse much less readily than control leaves of the same plant in $\frac{M}{2}$ urea.

Experiment 2. A. $\frac{N}{40}$ NaOH + 0.75 m C_2H_5OH in tap water —decolorized in ten minutes.

B. $\frac{N}{40}$ NaOH + 0.37 m C_2H_5OH in tap water—decolorized in 19 minutes.

C. $\frac{N}{40}$ NaOH in tap water—decolorized in 19 minutes.

D. 0.75 m C_2H_5OH in tap water—rotation momentarily accelerated, then slowed and continued slow for $>$ 1 hour.

E. 0.37 m C_2H_5OH in tap water—rotation hardly affected, slightly accelerated if anything.

Experiment 3. A. $\frac{N}{40}$ NaOH + 0.075 m NaCl in distilled water—decolorized in 4 minutes.

B. $\frac{N}{40}$ NaOH in distilled water—decolorized in 28 minutes.

C.   0.075 m NaCl in distilled water—rotation unaffected after 24 hours.

Experiment 4.   In this experiment the effect of salts, and the penetration of NaOH into red stained Elodea and also Spirogyra were studied.   The salt solutions are all in distilled water.

	SPIROGYRA	ELODEA
	min.	*min.*
$\frac{N}{40}$ tap water NaOH	30	45
$\frac{N}{40}$ distilled water NaOH	10	35
$\frac{N}{40}$ NaOH + $\frac{N}{10}$ NaCl	$\frac{1}{4}$	8
$\frac{N}{40}$ NaOH + $\frac{N}{10}$ (100 NaCl + 1 CaCl$_2$)	3	9
$\frac{N}{40}$ NaOH + $\frac{N}{10}$ (100 CaCl + 2.2 KCl)	$\frac{1}{4}$	5
$\frac{N}{40}$ NaOH + $\frac{N}{10}$ (100 NaCl + 2.2KCl + 1.6 CaCl$_2$)	2	6

Experiment 5.

	SPIROGYRA	ELODEA
	min.	*min.*
$\frac{N}{40}$ tap water KOH	30	35
$\frac{N}{40}$ distilled water KOH	10	30
$\frac{N}{40}$ KOH + $\frac{M}{8}$ glycerine in distilled water	10	19
$\frac{N}{40}$ KOH + $\frac{M}{4}$ glycerine in distilled water	5	14
$\frac{N}{40}$ KOH + $\frac{M}{8}$ urea in distilled water	5	19
$\frac{N}{40}$ KOH + $\frac{M}{4}$ urea in distilled water	5	14

Both the chloroform and alcohol in sufficient concentration allow the more ready entrance of NaOH (experiments 1 and 2). One-sixth saturated chloroform also retards plasmolysis by $\frac{M}{2}$ urea.   Such a retardation must be due to the fact that urea can enter chloroform cells more readily than normal cells.   Urea penetrates normal cells slowly.

A most striking effect is exerted by the neutral salts on the penetration of NaOH (experiments 3 and 4), especially with Spirogyra.   A concentration which may enter in distilled water only after 10 minutes, passes the cell membrane instantly in $\frac{N}{10}$ NaCl.   The effect on Elodea is similar.   Addition of CaCl$_2$ prevents the ready permeability to NaOH[8].   In Spirogyra the

[8] No precipitate of CaCO$_3$ is formed.

action is not marked but it is constant. It suggests that the effect of the pure NaCl is on the membrane, not on the NaOH.

Lillie ('11) has held, and especially emphasizes this in a recent paper, that the action of a pure isotonic solution is to increase the permeability of the cells exposed to the action, and antitoxic cations as Ca, prevent such an increase to a certain extent.

4. Experiments with animal cells

a. Paramoecium: Although several observers have investigated the toxicity of and physiological effect of the inorganic alkalies, no study of their power of penetrating animal cells has as yet been made. It is generally assumed in consequence of marked functional alterations produced that the cell is readily permeable for them. On the contrary the permeability relations have turned out to be exactly similar to those of plants. The same two classes of alkalies may be recognized, the weak (NH_4OH and amines) and the strong (inorganic hydroxides and $N(C_2H_5)_4$-OH), the former meeting a very slight resistance, if any, the latter a marked resistance.

Neutral red was again made use of as an indicator. The Paramoecia were stained in a watch glass by adding just enough of the dye so that it is practically all taken up by them from solution. No abnormalities or functional changes appeared. If an excessive amount of neutral red is added the organisms cytolyse in a manner typical of NH_4OH (see p. 543).

The fact that Paramoecium has a mouth through which alkali may enter introduces no error into the experiments, for the mouth is not open but the point at which vacuoles form is protected by a surface film. Its osmotic properties are unknown but most probably are essentially similar to those existing over the rest of the cell. The course of an experiment is often very short and the alkali is dissolved in distilled water in which there is no food to be eaten.

The change undergone by Paramoecium in alkalies is very similar to that which occurs when in the presence of a great many other toxic substances (alcohol, chloroform, chloretone, nicotin

and other alkaloids, KCN and lack of oxygen (Budgett '98) and has been designated as cytolysis. An excellent account of the process is given by Wulzen ('09).

The essential effect is a change in shape, with the protrusion of clear drops (vesicles) from the surface and the separation of a more or less well defined membrane. On account of the change in shape (shortening and widening) it is difficult to say how much swelling accompanies cytolysis.

The exact changes vary somewhat according to the alkali and the strain of Paramoecium used, but the sequence is fairly constant, as follows:—

Motor reflex or avoiding reaction.

Change in shape.

Swim backward (not always observed).

Color change appears (with NH_4OH).

Swim slowly in circles without making headway.

Clear drops appear at surface.

Swimming ceases.

Drops fuse to a membrane.[9]

Surface bursts.

Color change appears (with NaOH).

At a certain point the power of swimming is lost and at another point the red is changed to yellow and diffuses out of the cell. The relative times for these two events to take place is given in table 6.

One drop of stained Paramoecia was mixed with 10 cc. of the alkaline solution in Syracuse watch glasses. A glass plate

[9] 'Membrane' is used in rather a broad sense. The type of membrane depends on the Paramoecium and on the alkali used. In $Ca(OH)_2$ the clear drops extruded rarely fuse and no definite membrane forms. In $Ba(OH)_2$ or NaOH an irregular membrane may form but I have never observed cilia beating on it. In NH_4OH a very definite membrane is lifted off, which must be the original surface film of the animal for the cilia may be seen beating on it. The original surface of Paramoecium within the membrane lifted off (in NaOH) is often perfectly clear and distinct and trichocysts may be seen beneath it. Lifting off of the whole ectoplasm plus pellicle by the accumulation of liquid beneath, occurs also in NaOH. Where this does not take place we must regard the membrane formed as either the pellicle or a haptogen film on the surface of the clear drops. The fact that this film is impermeable for NaOH points to the former alternative.

excluded dust and prevented evaporation. The water was redistilled in glass from $K_2Mn_2O_8$ and NaOH and was non-toxic. The first third of the distillate was rejected.

Two different cultures of Paramoecia were used and they showed characteristic differences, both as regards resistance to the toxic effect of the alkali and rate of penetration of the alkali. Different species of Protozoa show likewise quite different degrees of resistance. An Oxytricha, Chilomonas and a Colpidium introduced along with Paramoecium into certain of the alkaline solutions appeared quite unaffected while the Paramoecia themselves were killed in a short time.

The individuals used in experiments, the results of which are given in table 6, were large and the amines and ammonias penetrated much less readily than in the second culture. The comparative differences between the alkalies are constant however.

In every instance the last seven substances enter Paramoecium readily and change the red dye to yellow; the first seven only enter long after all motion has ceased, and the organism is very much swollen and dead. If it burst or is crushed so that the surface is ruptured the alkali enters at once and turns the neutral red to yellow. Or if the animal's surface is changed by $CHCl_3$ water or chloretone the alkali is found to enter immediately.

A detailed comparison of the effects produced by NH_4OH and NaOH on stained Paramoecium will serve to make clear the differences between the two groups of alkalies, as regards diffusibility through cell membranes. The second culture of smaller individuals was used in this comparison.

$\frac{N}{1000}$ NH_4OH—Paramoecia placed in this solution at first give the avoiding reaction. The movement is immediately slowed and the animals revolve slowly on their long axis first forward a short distance then backward. Change in shape begins immediately, the twist of the hind end becoming less marked. The change in color of neutral red also begins immediately; the red gradually fades and the animals are colorless in two to three minutes. Some individuals showed clear drops (vesicles) along the sides of the body in about four minutes. These individuals ceased movement in five minutes and disintegrated. The remainder (about half) simply swell often with protrusion of the

TABLE 6

CONCENTRATION OF SOLUTION		$\frac{N}{320}$	$\frac{N}{640}$	$\frac{N}{1280}$	$\frac{N}{2560}$	$\frac{N}{5120}$	$\frac{N}{10240}$	$\frac{N}{20480}$
NaOH	movement ceases	4 minutes	45 minutes	normal and red after 6 hours	same	same	same	same
	color change	10 minutes	90 minutes	normal after 24 hours				
KOH	movement ceases		30 minutes	normal, red after 6 hours	same	same	same	same
	color change		70 minutes	normal after 24 hours				
Ca(OH)₂	movement ceases		20 minutes	swell within 1 minute; swell within 1 hour; recovering and red after 6 hours a few dead; most living after 24 hours	normal and red after 6 hours; normal after 24 hours	same	same	same
	color change		65 minutes					
Sr(OH)₂	movement ceases		2.5 minutes	swell within 1 minute very slow in 1 hour many dead within 2 hours; a few living and red after 6 hours and living after 24 hours	normal and red after 6 hours; normal after 24 hours	same	same	same
	color change		6 minutes					
Ba(OH)₂	movement ceases		2.5 minutes	most swollen and dead after 2 hours. All dead after 6 hours	normal and red after 6 hours; a few dead but most living and red after 24 hours	living after 24 hours	same	same
	color change		6 minutes					
N(C₂H₅)₄OH	movement ceases	5 minutes	normal after 4 hours					
	color change	7-8 minutes						

[10] A third strain of Paramoecia were tested with $N(C_2H_5)_4OH$, which was only obtained after the experiments with the other alkalies of table 6 had been completed.

Substance	Effect					
NH₄OH	movement ceases	5 minutes	50 minutes		normal and color-less after 6 hours normal after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	3 minutes	6 minutes		same	same
NH₃(CH₃)OH	movement ceases	2 minutes	12 minutes	30 minutes	swollen and color-less after 1 hour living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	2 minutes	6 minutes	12 minutes		
NH₂(CH₃)₂OH	movement ceases	1 minute	7 minutes	30 minutes	slightly swollen and colorless after 1 hour; living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	1 minute	5 minutes	15 minutes		
NH(CH₃)₃OH	movement ceases	1.5 minutes	9 minutes	30 minutes	swollen and color-less after 1 hour; living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	1.5 minutes	5 minutes	12 minutes		
NH₃(C₂H₅)OH	movement ceases	2 minutes	8 minutes	30 minutes	swollen and color-less after 1 hour; living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	2 minutes	6 minutes	10 minutes		
Normal propylamine	movement ceases	1 minute	6 minutes	30 minutes	swollen and color-less after 1 hour living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	1 minute	4 minutes	10 minutes		
Isopropyl-amine	movement ceases	1.5 minutes	7 minutes	30 minutes	slightly swollen and colorless after 1 hour; living after 24 hours	normal and red same after 6 hours; normal after 24 hours
	color change	1.5 minutes	4 minutes	8 minutes		

ectoplasm bearing cilia, but swim about normally although slowly for over fifteen minutes. A much more rapid rate of swimming is regained soon after the slowing, which takes place when first subjected to the action of the solution.

$\frac{N}{500}$ NaOH—Paramoecia placed in this solution swim rapidly forward but in less than one quarter minute they stop short and give the avoiding reaction. Many stop suddenly and swim rapidly backward. Change in shape begins immediately and in three minutes they are much shorter and broader and move slowly forward, circling about their long axis. In four minutes clear drops (vesicles) and also clear protrusions appear on the surface. The protrusions may contain many red stained granules. After eight minutes some individuals have burst and the neutral red, which up to this time has been red, immediately turns yellow. Most of the Paramoecia burst before twelve minutes but a few are still moving very slowly and appear as red as when first placed in the solution.

Essentially, the series of changes undergone in the two solutions is the same. The NH_4OH meets no resistance at the surface and the red color may be seen to change gradually to yellow, which diffuses out leaving the organisms colorless, from the moment they are placed in the solution. The change is complete before droplets appear at the surface or movement ceases. NaOH does not enter until after movement has ceased and the organism is enormously swollen and has lost all semblance to its original shape. Red granules may be present directly under the surface yet they remain red. Once the NaOH does begin to enter it does so rapidly and what is left of the Paramoecia becomes first yellow then colorless, in less than two minutes from the time the alkali begins to pass in.

We must draw the conclusion, that the NaOH produces the changes in behavior, the vesicle formation, the cessation of movement and the final death of the animal, all by *an effect on its surface membrane.* So long as NaOH alone was studied I could never be sure that a small amount of NaOH, too small to affect the red stained granules, did not enter and was not responsible for the observed changes. But the comparison with NH_4OH shows how low the OH ion concentration that is required to decompose the red granules really is provided the alkali may enter

freely. Ammonia, likewise, must affect the membrane in time since it produces change in behavior, vesicle formation, cessation of movement and finally death, but the changes produced bear no relation to the time of entrance.

Even in very dilute solutions NH_4OH and the amines are able to 'decolorize' stained Paramoecia but it takes a longer time. Yet in equivalent molecular concentrations of KOH, NaOH, etc., the animals sometimes retain their red color for 24 hours. Generally they are found to be colorless in that time. The decolorization is not due to the slow entrance of alkali because the same red individuals placed in distilled water are found to be colorless after 24 hours, although otherwise unchanged. A comparison after a shorter time, six hours, must be made. The results are given in table 6.

NH_4OH enters the cell readily and sets free a small amount of the neutral red base from its granule combination. The freed base diffuses out into the medium and more NH_4OH enters. Thus the process is repeated until the organism is quite colorless. That the same decolorization does not take place in KOH must be due to the fact that the KOH does not enter freely.

In very weak concentrations ($\frac{N}{10240}$) NH_4OH fails to affect the red color of stained Paramoecia at all. This concentration is presumably below the limit necessary to free the neutral red from its combination with the granules.

b. Marine eggs: In the following experiments the eggs of both Toxopneustes and Hipponoë were used. The sea water at Boca Grande,[11] where the experiments were performed is markedly alkaline to neutral red and faintly so to phenolpthalein. If Toxopneustes eggs, unfertilized, are placed in 100 cc. sea water + 1.2 cc. $\frac{N}{16}$ NaOH sufficient alkali does not enter them to turn the neutral red yellow for over three hours. If chloroform is added to the sea water, the eggs almost instantly turn yellow. Chloroform likewise causes the eggs to swell (cytolysis), an effect prevented in plant cells by the presence of a cellulose wall, and the penetration of the alkali might be connected with the swell-

[11] About twelve miles west of Key West and sixty miles east of Tortugas.

ing. The following experiment in which cane sugar is added to the sea water shows that in the presence of chloroform the alkali may enter the eggs before swelling of the egg has begun. Sugar prevents rapid pushing out of the artificial fertilization membrane which is relatively impermeable to it.

A. 10 cc. (50 cc. sea water + 10 cc. 2 m cane sugar) + 0.15 cc. $\frac{N}{10}$ NaOH saturated with cholorform.

Neutral red stained eggs are turned yellow in 3–4 minutes. After about 5 minutes swelling is noticeable. If an acid is added the eggs are turned pink again.

B. Control (10 cc. (50 cc. sea water + 10 cc. 2 m cane sugar) + 0.15 cc. $\frac{N}{10}$ NaOH.

Red stained eggs remain red for over three hours, in the meantime undergoing irregular division and fragmentation. Even very small fragmented spheres retain their red granules intact, providing their surface is likewise intact.

Just as in Paramoecium, observation of the manner in which the color change occurs points to the view that the alkali only enters after it has destroyed the surface. In immature Pentaceros eggs the red staining granules are present at the periphery separated from the alkaline solution only by the surface film of the egg. Yet they remain red for 15 minutes in $\frac{N}{320}$ NaOH in 0.6 n NaCl. Once the NaOH begins to enter the color change is very rapid and the egg swells simultaneously.

The same is true of Toxopneustes where the red granules are uniformly distributed. There is never a gradual entrance of alkali from the moment the eggs are placed in the alkaline solution but after a certain interval the NaOH passes the surface and then it may be seen to move rapidly within the egg.

Toxopneustes eggs even undergo irregular division in hyperalkaline sea water (100 cc. sea water + 1.3 cc. $\frac{N}{10}$ NaOH) without the entrance of enough alkali to change the red to yellow. I have not experimented with NH_4OH but it is probable, judging from my Paramoecium experiments, that this alkali would pass into the eggs freely and induce the color change before division, cytolysis, or any injury to the egg takes place. If such were the case it would show that the action of NaOH as a parthenogenetic

agent must be on the egg surface alone and not the catalytic acceleration of any reactions within the egg through an excess of OH ions.

A resistance to the entrance of NaOH is likewise shown by the eggs of Holothuria Floridana, Hipponoë esculenta, Pentaceros, reticulatus, and Asterias vulgaris.

A comparison of the entrance of alkali into fertilized and unfertilized eggs has shown that the fertilized eggs of Toxopneustes are much more readily entered by NaOH just after fertilization and again about the time of first cleavage. Only one experiment was performed toward the end of the breeding season and the eggs of the female used cleaved in the control in many instances somewhat irregularly. A stock solution of $\frac{N}{320}$ NaOH in 0.5 m NaCl was made up. The unfertilized and fertilized eggs at intervals after fertilization were compared with each other as to entrance of NaOH, in separate watch glasses over a white background. It is thus easy to see when all the eggs have been entered by the alkali, and their original red color changes to yellow. The following table shows the result.

TIME AFTER FERTILIZATION	TIME TO TURN FERTILIZED EGGS YELLOW	TIME TO TURN UNFERTI- LIZED EGGS YELLOW
min.	min.	min.
2	13	19
5	14	21
10	19	19
20	20	22
30	21	21
45	17	20
55	21	21
65	20	20

The eggs begin to cleave about 45 minutes after fertilization.

A somewhat similar result is obtained by comparing mature Asterias eggs fertilized and unfertilized, as well as eggs treated with acetic acid. The latter form artificial membranes. The results are shown in the following experiment.

	$\frac{N}{160}$	$\frac{N}{320}$ NaOH in 0.6 m NaCl.
	min.	*min.*
Fertilized eggs (4 minutes after fertilization).........	5	15
Fertilized eggs (20 minutes after fertilization)..........	6	20
Unfertilized eggs (control).............................	11	30
Unfertilized eggs (5 minutes after acetic treatment)...	6	20

Contrary to the results obtained in the Toxopneustes experiment it will be noted that the starfish eggs did not regain their original resistance to NaOH a short time after fertilization.

While it is of course true that in time NaOH may enter sea urchin eggs as well as Paramoecium or plant cells, and in this sense they are difficultly permeable, it seems better, in considering the *inorganic alkalies and* $N(C_2H_5)_4OH$, to speak of a *resistance* of the cell against their entrance rather than a *permeability* of the cell for alkalies as I have done in a preliminary report ('10). The change undergone at the time of fertilization results in a surface less resistant to the penetration of alkali. At the same time the decrease in resistance for alkali is presumably connected with an increases in permeability for other substances notably the salts of sea water, as indicated by the experiments of McClendon ('11) and Lyon ('10).

In working with Elodea (p. 539) I was able to show that small concentrations of chloroform which inhibited the protoplasmic rotation, but without any irreversible changes, increased the rate with which NaOH entered the cells. Exactly the same fact may be shown for the sea urchin's egg as the following experiment indicates. Unfertilized Hipponoë eggs stained in neutral red were placed in these solutions.

A. $\frac{N}{320}$ NaOH, $\frac{1}{4}$ saturated with chloroform, in $\frac{5}{8}$ m NaCl.
B. $\frac{N}{320}$ NaOH in $\frac{5}{8}$ m NaCl.
C. $\frac{1}{4}$ saturated chloroform in $\frac{5}{8}$ m NaCl.
D. $\frac{N}{320}$ NaOH, $\frac{1}{4}$ saturated with ether, in $\frac{5}{8}$ m NaCl.
E. $\frac{1}{4}$ saturated solution of ether in $\frac{5}{8}$ m NaCl.

In solution A the alkali has turned the eggs yellow in 10 minutes, in D in 6 minutes and in B in 20 minutes. Eggs in the chloroform

control, C, were uncytolyzed in one hour and about one-half of them cytolyzed in the course of two hours. Eggs of the ether control, E, were unaffected in 30 minutes and one-half of them cytolyzed in 45 minutes.

DISCUSSION

In an extensive paper, Barratt ('04) has studied the action of both acids and alkalies on Paramoecium; my results on alkalies are in fair quantitative agreement with his. Barratt came to the conclusion that neither the alkali nor acids produced their effect by a catalytic splitting of any substances in the organism but by a combination of the acid and alkali with the protoplasm. It was proved that the concentration of acids and alkali decreases in solutions in which a large number of Paramoecia had been placed. Three methods of determining this were used, viz.: (1) the use of an indicator, (2) measuring the electrical conductivity, (3) ('05) determining the E. M. F. by means of hydrogen electrodes. Only a relatively small amount of acid and alkali combines. One hundred parts of living Paramoecium take up 0.25 parts of HCL and 1.5 parts of NaOH.

I fully agree with Barratt that the toxicity of the alkalies bears no relation to the OH ion concentration. The order of toxicity for Paramoecium is $N(C_2H_5)_4OH$ (?) $<$Na or K or Ca $<$Sr or Ba $<NH_4$ $<$amines. The degree of dissociation is NH_4 $<N$ $(CH_3)_3H$ $<$primary and secondary amines $<N(C_2H_5)_4$$<$Ba, Sr, Ca, K, Na.

Comparison of all the alkalies above mentioned in regard to toxicity is hardly legitimate since the two distinct classes, the strong and the weak, differ so in their power of penetrating the cell. It is to the rapid entrance of the weak alkalies, that their greater toxicity and general difference in physiological action is to be referred.[12] But even among the strong alkalies, equally

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[12] Mathews (American Journal of Physiology, vol. 18, p. 58, 1907) states that the nucleolus of Asterias eggs immediately disappears from view in NH_4OH and $(NH_4)_2CO_3$ but not in NaOH or Na_2CO_3. This is possibly due to solution in the entering NH_4OH. The NaOH cannot enter and no solution takes place. The carbonates are hydrolytically dissociated into NH_4OH and NaOH respectively.

dissociated in dilute solutions, there are characteristic specific differences, as a comparison of Ba and Na will indicate. These two substances differ markedly in toxicity even though they both fail to pass the cell surface.

On the other hand, Barratt's conclusion is not in accord with the observation of Paul and Kronig ('96) who found Na, Li, and KOH equally toxic to bacterial spores and NH$_4$OH less so. Loeb ('97) also found that the increase in weight of muscle caused by Li, Na, K, Sr, and Ba hydroxides depends on the OH ion concentration.

As previously stated the power of penetration, in general, bears no relation to the toxicity except among the strong alkalies which fail to enter until the cell is fatally affected. Among the weak alkalies NH$_4$OH enters most rapidly yet is less toxic, than methyl or dimethyl amine.

The most apparent relation is between the rate of entrance and the degree of dissociation. The weak alkalies enter rapidly, the strong very slowly. N(C$_2$H$_5$)$_4$OH is an excellent confirmation of the rule. A priori it was to be expected that the tetra-alkyl substitution product of NH$_4$OH would behave just as its primary, secondary, and tertiary derivatives. Tetraethylammonium hydroxide should enter cells rapidly yet such is not the case. The substitution of four C$_2$H$_5$ groups for four H atoms gives a substance whose degree of dissociation may be compared with the strongest inorganic bases, Na or Ba. Correspondingly its power of penetrating cells is likewise limited and comparable with that of Na or Ba.

The general relation between the dissociation or chemical reactivity of the alkalies and their power of penetrating cells suggests that, while we appear to be studying cell permeability, we are in reality confronted with phenomena of reaction velocity, depending on the strength of the base. NH$_4$OH may appear to penetrate readily, because, being a weak base, it combines with the cell proteids less rapidly and so may affect the neutral red. NaOH appears to meet a resistance because it combines readily with the cell proteids as it enters and cannot affect the neutral red. But in both cases the proteids must be acted upon before the

indicator is affected. Were the relation of Na and NH$_4$ reversed, some probability for such an explanation might be found. The evidence (p. 528) that combined NH$_4$OH or NaOH albumen does not affect neutral red even in solution, that chloroform has no influence on the combining power of albumen for alkali, whereas cells killed by chloroform become as readily permeable for NaOH as for NH$_4$OH is strong proof that permeability and not reaction velocity is in reality the phenomenon studied. Robertson's ('10) experiments on the solution of casein in alkalies indicate that equal concentrations of Na, K, Li, and NH$_4$ dissolve equally well. Even though the velocity of solution is mainly due to the rate of wetting of the casein particles by the alkali, the analogy is all the closer to diffusion into a cell and any marked differences in NH$_4$OH and NaOH should appear.

Lastly, it must be pointed out that the rate of penetration is not exactly inversely proportional to the degree of dissociation. Among the weak alkalies trimethyl amine is much less dissociated than methyl amine yet enters Elodea less rapidly.

Overton's study of the penetration of the weak organic bases into Spirogyra cells as indicated by precipitation of a tannate has brought out the relation which exists between lipoid solubility and permeability. According to Overton, ammonia and the amines with the exception of the quaternary ammonium bases are lipoid soluble. The two classes into which we may divide the alkalies in respect to their power of penetrating living cells are just the classes into which we may divide the alkalies in correspondence to Overton's hypo hesis. Traube has shown however that the lipoid solubility of a substance is also connected with a lowering of the surface tension of water by that substance. It is to this property that he refers the ready penetration of lipoid soluble substances, in accordance with his theory of osmosis. It is quite probable that the relations shown by the alkalies may be brought into line with Traube's theory but I have no data on the subject and cannot at present discuss the question.

I also agree with Barratt that NaOH combines with certain constituents of Paramoecium but would limit the combination

to the surface layer only. This would explain the relatively small amount of alkali which Barratt found to be taken up.

The change thus brought about in the cell surface alters its properties in three conceivable directions,—surface tension, electrical polarization and permeability. The alteration in the latter property is in the direction of an increase in permeability, since finally the surface becomes so altered as to allow the inorganic alkali itself to pass.

In Paramoecium, cytolysis, in Elodea, rotation, and in the sea urchin egg, irregular fragmentation and division result before any appreciable amount of NaOH has entered. We must consider then, that all these changes are essentially an expression of some profound alteration in the cell surface, involving one or all of the properties mentioned above.

In recent years more and more stress has been laid on the importance of a surface layer differing markedly from the interior of the cell and the role played by surface energy in cell dynamics. It seems that we cannot attribute too much functional value to this barrier or passageway between cell and environment, the 'Umwelt und Innenwelt.' Life has been defined as "the continuous adjustment of internal relations to external relations." Should we not, therefore, look to the boundary between the two 'worlds' for an explanation of many of the phenomena exhibiting such unusual characteristics as to receive the special designation —vital?

SUMMARY OF RESULTS

1. The basic dyes fail to enter cells in the acid condition, i.e., as the dye salt. Certain acid dyes do enter in the acid condition but not in neutral or alkaline solution. The latter combine with the nucleus and protoplasm leading to death of the cell; the former (in the concentrations used) with non-essential elements. In animal cells, these elements are granules which may be distinguished by their specific gravity. As a general rule they are the heaviest substances present, as determined by the centrifuge.

2. Using neutral red as an indicator within the cell it was found that both animal and plant cells behave similarly with respect to the penetration of organic and inorganic hydroxides. Two classes may be recognized, the strong alkalies ($N(C_2H_5)_4$-OH, Na, K, Ca, Ba, and Sr hydroxides) and the weak (NH_4OH and the amines). The former enter with difficulty and only after destroying the normal properties of the surface; the latter, readily, and independently of such surface action. Both classes penetrate dead cells rapidly. Difference in physiological action is to be referred to their respective powers of penetration.

The inorganic alkalies produce very marked functional changes without appreciably penetrating the cell. Such action must be attributed to an alteration of the surface layer. Functional changes cannot therefore be used as a criterion of permeability.

Decrease in the resistance of the cell surface may also be brought about by quantities of ether, alcohol, chloroform, and various salts too small to produce any irreversible effects. Normal variations in resistance also occur among cells apparently in the same condition of functional activity or between cells known to be in different conditions of activity (the unfertilized as compared with the developing egg).

Considering all the alkalies studied, there is no relation between toxicity and penetrating power. This is likewise true for the class of weak alkalies. Those strong alkalies are most toxic which most readily destroy the resistance of the plasma membrane. Hence, using permeability in the broad sense, the most toxic of the strong alkalies penetrate the cells most rapidly. The two classes support Overton's hypothesis in their lipoid solubility and penetrability relations and probably also that of J. Traube.

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THE EFFECT OF EXCRETION PRODUCTS OF PARAMAECIUM ON ITS RATE OF REPRODUCTION

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ELEVEN FIGURES

Evidence from many sources points to the fact that a considerable amount of the products of metabolism of a cell is frequently injurious to the cell. As is well known, alcohol in excess of a certain amount inhibits the reproduction of yeast, while the same amount forms a favorable medium for the organism which produces vinegar. Again, vinegar in excess of a certain quantity is toxic to the cells which are responsible for its formation, but serves in turn as a favorable environment for organisms which give rise to another type of fermentation.

De Candolle[1] nearly eighty years ago apparently suggested that low crop production, following continued growth of one crop in the same soil, is due to the accumulation in the nutrient medium of deleterious organic substances originating in the growing plants themselves. Liebig[2] also held this view for a time but later abandoned it in favor of the idea that the beneficial effect of crop rotation is due to the several crops requiring different substances or varying proportions of the same substances, and that the disturbance of the balance in the soil produced by one crop is not unfavorable to the growth of some other crop. The trend of much recent research is distinctly in support of the view that substances are produced by the higher plants which are speci-

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[1] DeCandolle, Physiologie végétale, Paris, 1832.
[2] Liebig, Complete works on chemistry, Familiar letters on chemistry, letter 12, p. 35, 1852. Cf. Gilbert, Bull. 22, Office Exp. Sta., U. S. Dept. Agr., 1895.

fically toxic to themselves and which form an unfavorable medium for the continued growth of a succession of the same species.[3] Recent work on various species of bacteria and molds points in the same direction. Eijkman,[4] for example, observed that these forms growing on artificial nutrient media form waste products which inhibit growth and also that these products of a given species are, as a rule, more toxic to that and closely related species than to those more distantly related. Further, Kuester[5] noted that molds grown in a solution produce substances which inhibit the growth of further inoculations.

Considerable experimental work on various vertebrates has demonstrated that fatigue is due to the accumulation of metabolic products. Ranke, and later Mosso[6] observed that the blood of fatigued animals when injected into the circulation of fresh ones brought about all the symptoms characteristic of fatigue, and Weichart[7] was able to isolate a toxin from fatigued muscle which, when injected into animals, gave rise to similar conditions.

Semper[9] in 1874 made a series of experiments with snails, in which he found, for example, that snails bred singly in two liters of water attained to more than three times the size of those grown in groups of twenty in the same amount of water. In all the experiments the amount of available food was maintained at the optimum. In other experiments the number of snails was constant and the volumes of water unequal, and the same result was obtained. Semper concluded that the results were in some

[3] Cf. Schreiner and Sullivan, Journ. Biol. Chem., vol. 6, pp. 39–50, 1906, and later papers; also Cameron, Journ. Phys. Chem., vol. 14, p. 425, 1910, and U. S. Farmers' Bull., 257, 1906.

[4] Eijkman, Centralbl. f. Bakt. 1, 37, p. 436, 1904. Cf. also Rahn, Centralbl. f. Bakt. 2, 16, p. 417, 1906.

[5] Kuester, Ber. d. d. Bot. Gesell., Bd. 26a, p. 246, 1908.

[6] Ranke, Tetanus: Eine physiologische Studie. Leipzig, 1865. Mosso, Arch. f. Anat. u. Physiol., Physiol. Abth., p. 89, 1890.

[7] Weichart, Münch Med. Wochenschr., Bd.f 2, p. 2121, 1904.

[8] Cf. Lee, Journ. Amer. Med. Assn., vol. 46, 1906, and Slade, Journ. Physiol., 35, 1907.

[9] Semper, Arb. a.d. Zool-Zoot. Inst., Würzburg, Bd., 1, 1874. Animal life, pp. 159–167, 1879.

way due to the volume of water available for each snail, but he was unable to determine how the volume produced the effects noted.   The same question was considered in 1894 by De Varigny[10] with considerably elaborated methods, and he found that the size of the snails was affected by the number of individuals in a given volume of water, but he did not observe that they were so markedly sensitive to differences in volume of the water. He concluded that the area of the surface exposed to the atmosphere had more influence than the volume.   Snails were bred by Whitfield[11] in a small aquarium for four generations and he found that the size of the shell decreased during each succeeding generation and that various other morphological changes occurred.

Yung[12] in 1885 made experiments with tadpoles and found that, within limits, the larger the area of the exposed surface of the water in which they were bred the more rapid their growth took place.   He concluded that the result was due to the water absorbing a larger proportion of oxygen from the air.

An extensive series of experiments was made by Vernon,[13] in 1895, in which, for example, he allowed eggs of several species of sea-urchins to develop in water which had previously been the environment for a considerable time of another batch of eggs, and he found that in every case the larvae of the second batch were diminished in size as compared with the control.   He concluded that products of metabolism excreted by the first batch retarded the growth of the second.   Other experiments apparently showed that the growth of larvae was decreased by their own metabolic products, and that the products of excretion of adult echinoids acted more adversely both on the life and on the growth of embryos if these belonged to the same species than if they belonged to other species—in fact he actually found that the

[10] DeVarigny, Journ. de l'Anat. et de la Physiol., p. 147, 1894.   Experimental evolution, pp. 79–88, 1892.

[11] Whitfield, Bull. Amer. Museum Nat. Hist., vol. 1, p. 21.   Amer. Naturalist vol. 14, p. 51..

[12] Yung, Arch. des Sci. Phys. et Nat. T. 14, p. 502, 1885.

[13] Vernon, Mittheilungen a.d. Zool. Sta. z. Neaple, 13, p. 389 et seq. Proc. Royal Soc. London, vol. 186, B. p. 603, 1895.   Variation in animals and plants, 1903.

excretion products of two less closely related species were favorable to growth.

More recently, Warren,[14] working with Daphnia magna, noted that, by continued breeding in small aquaria in which the water remained unchanged, the rate of reproduction and the number of young in a brood was markedly decreased, and also that the length of the spine of the carapace was considerably shortened. This result he attributed to the excretion products of the daphnids and, since ostracods and copepods flourished when the daphnids were waning, he concluded that the products of metabolism of Daphnia are specifically injurious to Daphnia.

Little work has been done to determine the effects of limited volumes of culture medium on the reproduction of Infusoria. Balbiani[15] in 1860 briefly reported a single experiment on Paramaecium from the results of which he concluded that the organism must be in not less than two or three cubic centimeters of infusion for the greatest reproductivity to be realized. Kulagin,[16] studying the generations of certain infusoria with reference to 'senile degeneration,' suggested that this was due largely to the fact that when the organisms have lived for a number of generations in the same water, they have contaminated the water by the excretion of substances analogous to toxins, and these gradually accumulate until the nuclei are affected.

The chief products of the destructive metabolism of the infusorian are in all probability water, urea, carbon dioxide and various salts, and these are eliminated chiefly by means of the contractile vacuole. A considerable amount of experimental evidence as well as analogy with higher forms points to the conclusion that CO_2 is voided in appreciable quantities by the contractile vacuoles of the Infusoria. For example, Jennings[17] determined that an acid is present in the vicinity of active paramaecia which is not sufficiently strong to attack $CaCO_3$, but which will

[14] Warren, Quart. Journ. Mic. Sci., vol. 43, p. 212, 1900.

[15] Balbiani, Comptes Rendus de l'Academie des Sci., Paris, T. 50, pp. 1191–1195, 1860.

[16] Kulagin, Le Physiologiste russe, T. 1, pp. 269–279, 1899.

[17] Jennings, Journ. Physiol., vol. 21, pp. 258–321, 1897.

decolorize rosol, and he therefore concluded that carbon dioxide is excreted by the organisms in quantities sufficient to be detected with proper reagents. The work of Barratt[18] may also be mentioned in which he found that the daily carbon dioxide production of Paramaecium varies between 1.3 and 5.3 per cent of the weight of the protoplasm, the variations in amount being largely due to temperature and the food supply of the animals.

Beyond the fact that CO_2 is eliminated by the infusorian cell and chiefly by the contractile vacuole, there are comparatively few data as to the form in which other products of katabolism leave the animal. The so-called excretory granules found in practically all groups of Protozoa have been quite carefully studied in Paramaecium. They were early supposed by Entz and others, by analogy with higher forms, to represent concretions similar to uric acid crystals. Schewiakoff[19] claimed, as the result of a series of careful tests, that they consist of calcium orthophosphate, and, as calcium is one of the most abundant metallic elements in cells, some probability is attached to his conclusion, especially since Schaudinn[20] determined the presence of calcium and phosphoric acid in the excretory granules of Trichosphaerium. Rhumbler,[21] however, considering the excretory granules of various Infusoria concluded that they consist of uric acid, and Griffiths[22] believed that he had demonstrated by microchemical tests the presence of uric acid in the contractile vacuoles of several types of protozoa, including Paramaecium.

Rossbach[23] in 1872 observed that temperature affected the frequency of the contractions of the contractile vacuole and this was confirmed by Maupas[24] in 1883. The French author also computed the relative volume of the contractile vacuole and cell of several Infusoria and determined, for example, that at a temperature of 27° C. Paramaecium aurelia evacuates a quantity of

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[18] Barratt, Zeit. f. Allgen. Physiol., Bd. 5, pp. 166–172, 1905.
[19] Schewiakoff, Zeitschr. f.-wiss. Zool. Phys., Bd., 17, 1893.
[20] Schaudinn, Abhandl. d. k. Akad. d. Wiss., 1899.
[21] Rhumbler, Zeitschr. f. wiss. Zoolog., 1899.
[22] Griffiths, Proc. Royal Soc. Edinburgh, vol. 16, 1889.
[23] Rossbach, Arb. a.d. Zool.-zoot. Inst. Würtzburg, pp. 9–72, 1872.
[24] Maupas, Archiv. de zool. exp. et gen. (2), 1 p. 648, 1883.

water equal to that of the entire organism in forty-six minutes, and similarly Cryptochilum nigricans does the same in two minutes. In 1907 Kanitz[25] found that, within certain limits, a rise of 10° of temperature caused a doubling of the rate of contraction, while the results of Khainsky,[26] 1910, showed that in Paramaecium caudatum the vacuole contracted 2.86 times per minute at 16° C., 6 times per minute at 23° to 25° C., and 10 times per minute at 33° to 34° C., under the conditions of the experiments.

It is clear then that excretion products in the case of many organisms have a profound effect on cell division and growth, and it is also clear that under favorable conditions of food and temperature the Infusoria excrete considerable amounts of carbon dioxide, together with various other end-products of metabolism, which may reasonably be expected to be evident through biological as well as chemical tests.

The ordinary 'hay infusion' teeming with animal and plant life is a microcosm in which every organism may and probably does in some degree affect the well-being of every other organism present. Besides the obvious influence exerted by animals in feeding on other forms and by green plants through photosynthetic processes, one would expect the effects of organisms on their environment by the elimination of products of their metabolism, or excretion products, to be one of the most important. The interdependence of the organisms of a hay infusion is so complex that, taken as a whole, it is almost beyond the possibility of analysis, and accordingly the logical method of approach to the subject is to study the interaction of isolated organisms and small groups of organisms on themselves and on each other. The present paper presents the results which have been obtained from the study of the effects on the rate of reproduction of Paramaecium of:

1. Different volumes of culture medium;

2. Changing the culture medium at twenty-four hour and at forty-eight hour intervals;

3. Culture medium in which rich growths of paramaecia have occurred.

[25] Kanitz, Biol. Zentralblatt, Bd. 27, 1907.
[26] Khainsky, Archiv. f. Protistenkunde, Bd. 22, I, p. 1, 1910.

EXPERIMENTS

The organisms used in this work were from my pedigree cultures of Paramaecium aurelia and Paramaecium caudatum. The experiments were begun on July 24, 1910, when the P. aurelia culture was at the 1903d generation and the P. caudatum was at the 113th generation, and were concluded on October 4, 1910, when the P. aurelia culture was at the 2070th generation and the P. caudatum culture was at the 288th generation. Emphasis is placed on the fact that the animals which formed the subjects for the experiments had been under daily observation for over forty months in the case of aurelia (which was the main culture employed), and over three months in the case of caudatum (which was used in certain experiments for comparison). Consequently their rate of reproduction, and the exact conditions to which they had been subjected for nearly three and a half years, were known in the case of the main culture. Further, since the pedigree cultures were each originally started with a single individual, all the P. aurelia used in this work were 'sister cells,' and all the P. caudatum used were 'sister cells.' Therefore all the experiments were performed on the 'same protoplasm' of the respective species. Fig. 1 shows graphically the average daily rate of division of the four lines of the P. aurelia culture again averaged for each month of its existence to the time it was employed for this work.[27]

1. *The effect of different volumes of culture medium on the rate of reproduction of Paramaecium*

A series of four experiments, two of sixteeen days duration and two of twenty days duration were made with P. aurelia. In all the work it was found that sixteen to twenty days was the most suitable length of time for the experiments, because those of less than sixteen days appeared too short to give conclusive results, and in those which were extended beyond twenty days, the ani-

[27] For details of these cultures see Woodruff, Biol. Bull., 16, 4, 1909; Archiv f. Protistenkunde, Bd. 21, 3, 1911; and Jour. Morph., vol. 22, 2, 1911.
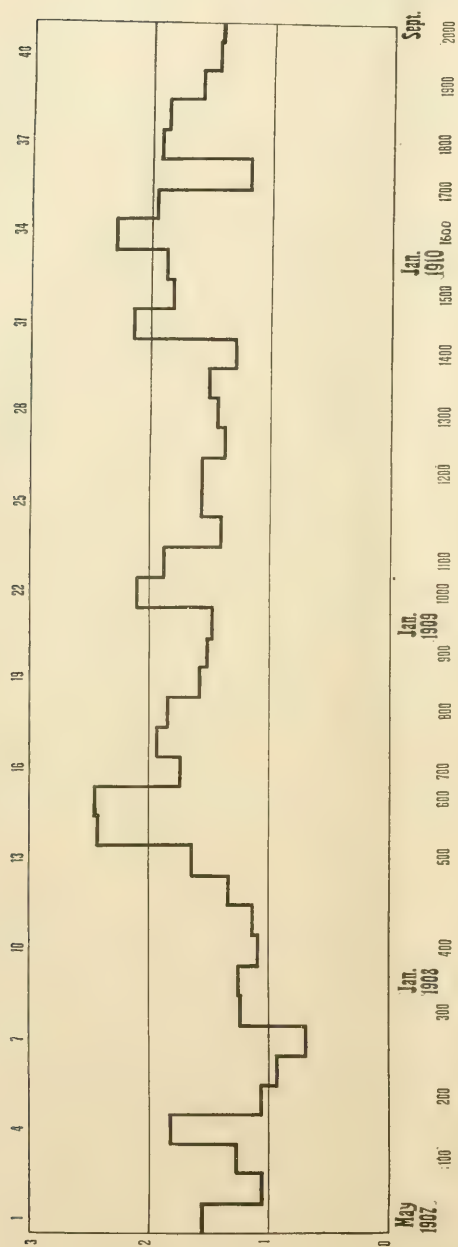
Fig. 1   Complete history of Paramaecium aurelia. Culture 1, from start on May 1, 1907, to September 21, 1910, at the 2000th generation. The ordinates represent the average daily rate of division of the four lines of the culture, again averaged for each *month* of the life of the culture to date.

mals on the various amounts of medium were about thirty genera-
tions apart, so that it might be suggested that this offered an ob-
jection.

The volumes of medium selected were two, five, twenty, and
forty drops.   The same pipet was used in measuring the liquid in
all the work, and so practical uniformity was attained.   Infusions
of hay were used as a culture medium and the organisms were
isolated on slides of different capacities, depending on the amount
of liquid employed.   The slides were kept in moist chambers to
prevent evaporation.

The daily rate of division of the organisms in two, five, twenty,
and forty drops of culture medium changed every *twenty-four hours*
showed that, for example, those in five drops divided 2.4 per cent
more rapidly than those in two drops, those in twenty drops
divided 6.4 per cent more rapidly than those in two drops, and
those in forty drops divided 7.4 per cent more rapidly than those
in two drops (fig. 4, part A).   The details of each of the four ex-
periments (A, B, C, D) are evident in fig. 2 which shows the rate
of division of each of the lines on the different amounts of medium
averaged for each four days of the experiment.   Experiments
B and C are the most important ones because these comprised
cultures on all the volumes of medium.   A was carried to test the
general method to be used, and D was carried to check up certain
data.

It is believed that the experiments are sufficiently comprehen-
sive clearly to establish the fact that *the rate of reproduction of
specimens from pure lines of paramaecia, when bred under identi-
cal conditions of temperature and culture medium, is influenced
by the volume of the culture medium (within the limits tested in the
experiments), and that the greater the volume the more rapid is the
rate of division.*

It being clear that in an increased volume of culture medium
there is an increased division rate, the next point of importance is
to determine to what factor or factors this is due.   It is evident
that it may be brought about by variations in 1, temperature; 2,
pressure; 3, surface of medium exposed to atmosphere; 4, food
supply; 5, excretion products of bacteria; or 6, excretion products
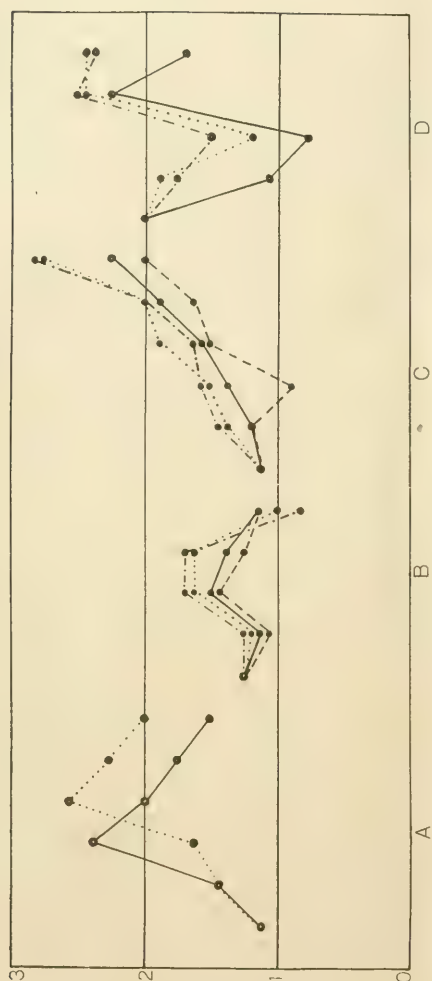of paramaecia.

Fig. 2 Record of the rate of division of Paramaecium aurelia in the series of four experiments (A, B, C, D) to determine the effect of different volumes of culture medium, changed every *twenty-four* hours, on their rate of reproduction. The ordinates represent the average daily rate of division of the four lines of organisms in the respective volumes of medium, again averaged for four day periods. Rate of division in two drops = - - - -, five drops = ——— , twenty drops = ..., forty drops = . — . — .

The experiments were conducted simultaneously and in the same place for all the volumes of medium, and therefore the temperature was the same.

It is believed that the exceedingly slight pressure variations in the different volumes of water was without appreciable effect. There is certainly no evidence extant that free-living protozoa are sensitive to such exceedingly small changes, and therefore this factor will be dismissed as not entering into the effects noted.

The question of increased surface exposure to the atmosphere in the larger volumes of media employed facilitating the exchange of gases, as oxygen and carbon dioxide, is possibly one of more importance, and accordingly care was taken to use receptacles of different capacities and shapes for the different volumes in an attempt to equalize the proportion of surface to volume of the different amounts of medium. Obviously it is practically impossible to make them actually equal, but certainly the precautions taken were sufficient to render this factor negligible.

The food supply is obviously a factor of great importance. The culture medium consisted of an infusion of hay which was raised to the boiling point to eliminate the possibility of contaminating the culture with 'wild' paramaecia, and was used after it had cooled. No precaution was taken to make the infusions exactly the same each time, but the same infusion was used for all cultures at the same time. This was made up fresh every forty-eight hours, beginning with the first day of each experiment, thus, in those experiments in which the medium was changed every twenty-four hours, the organisms were transferred to some of the medium still remaining in the stoppered flask from the day before. This flask was shaken before it was used, and there is every reason to believe that the organisms in each amount of medium received exactly the same food. Slight variations in the bacterial flora were avoided, it is believed, by the fact that at the beginning of each experiment all the paramaecia came from the same environment, and consequently the bacteria transferred with them when they were isolated would presumably be the same in each case, and if they were not the same, or if the new infections from the air, which must have occurred in the course of

Fig. 3 Record of the rate of division of Paramaecium aurelia in the series of four experiments (A, B, C, B,) to determine the effect of different volumes of culture medium, changed every *forty-eight* hours, on their rate of reproduction. The ordinates represent the average daily rate of division of the four lines of organisms in the respective volumes of medium, again averaged for four day periods. Rate of division in two drops = - - - -, five drops = ———, twenty drops = · · · ·, forty drops = · — · — ·

the experiment, tended to render the media of the lines different, this variation was eliminated by cross infections of all the lines of all the cultures daily at the time of isolation. It is believed then, that every practicable precaution was taken to ensure as near absolutely the same food conditions as it is feasible to obtain, and consequently that the results have not been influenced by variations in nutrition.

It is not apparent that the consistent variations in the division rate in the different volumes of medium can be the result of the products of metabolism of bacteria, in view of the great care taken to maintain identical flora in all the preparations. It is possible, however, that slight irregularities in the curve may be explained on this basis and this point will be mentioned later.

The remaining factor to be considered is the possible effect of the excretion products of the paramaecia themselves. That the infusoria in their ceaseless activity are continually voiding appreciable amounts of carbon dioxide and other products of metabolism is evident from the work of previous investigators. The question then is—is the amount excreted sufficient to affect the division rate appreciably under the conditions of the experiments? Taking the average rate of division during the experiments as one and one-half divisions per day, the average number of organisms in a preparation during the first twenty-four hours would be one and one-third—one during the first sixteen hours, and two during the following eight hours. However improbable it may seem that this number of individuals can excrete sufficient toxic substances to have an appreciable effect on the division rate when diluted, for example, by twenty drops or by forty drops,[23] I believe the experiments outlined make this highly probable, and point to the conclusion that *the variations in the daily division rate in the different volumes of water is due to the excretion products of the paramaecia themselves.*

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[23] With the division rate increasingly more rapid with increase of volume, there would actually be more excretion products in the larger volumes than in the smaller. This however can be disregarded.
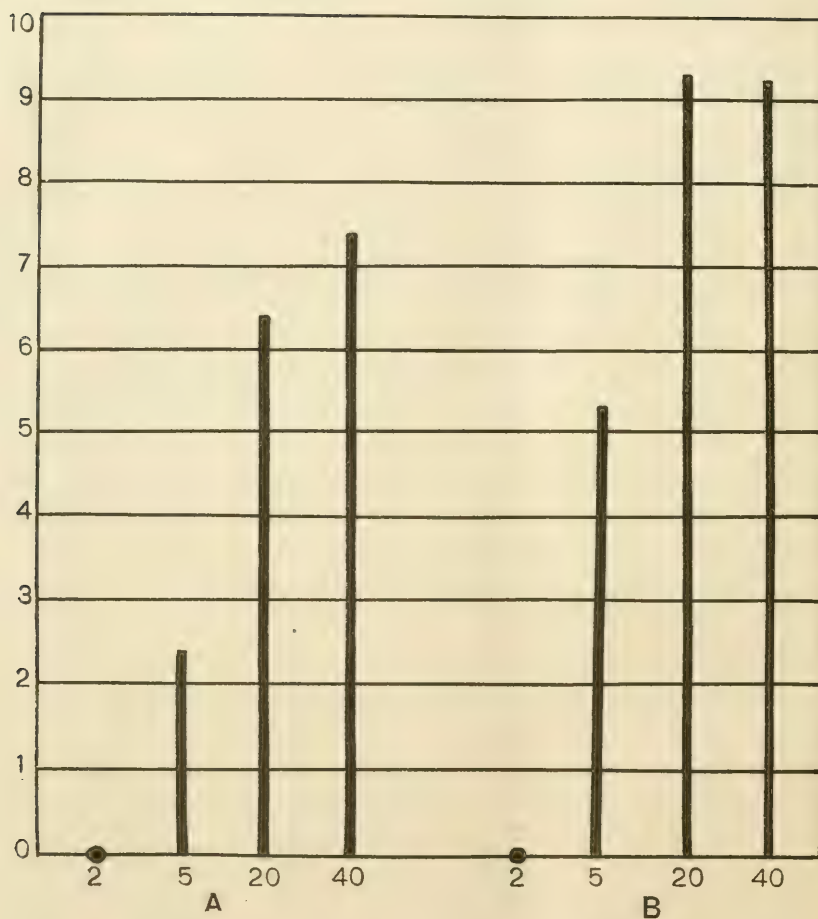
Fig. 4 Summary of the results of the experiments plotted in figs. 2 and 3. *A* shows the per cent gained in division rate by Paramaecium aurelia in five, twenty, and forty drops of medium changed at *twenty-four*-hour intervals, over the division rate of those in two drops changed also daily. *B* shows the per cent gained in the division rate by P. aurelia in five, twenty, and forty drops of medium changed at *forty-eight*-hour intervals over the division rate of those in two drops changed also on alternate days.

2. *Effect of changing the culture medium at twenty-four and forty-eight hour intervals, in each of the several volumes, on the rate of reproduction of Paramaecium*

If the conclusion reached from the previously outlined experiments is true, the effects of the excretion products should manifest themselves more clearly in cultures in which the organism remained in the medium a longer period, than in those in which the organism remained in the medium for a shorter period of time.   To test this a second series of experiments was carried out *simultaneously* with those already described, and in this second series the animals remained in the same medium for forty-eight hours instead of twenty-four hours.   There were, then, the following series of cultures involved in this experiment:

     Ad 2 = P. aurelia in 2 drops of medium changed daily.
     Ad 5 = P. aurelia in 5 drops of medium changed daily.
     Ad20 = P. aurelia in 20 drops of medium changed daily.
     Ad40 = P. aurelia in 40 drops of medium changed daily.
     Cd 5 = P. caudatum in 5 drops of medium changed daily.
     Aa 2 = P. aurelia in 2 drops of medium changed on alternate days.
     Aa 5 = P. aurelia in 5 drops of medium changed on alternate days.
     Aa20 = P. aurelia in 20 drops of medium changed on alternate days.
     Aa 40 = P. aurelia in 40 drops of medium changed on alternate days.
     Ca 5 = P. caudatum in 5 drops of medium changed on alternate days.

Each of these cultures comprised four separate lines, *e.g.*, *Ad2 −1*, *Ad2–2*, *Ad2–3*, and *Ad2–4*, and the number of divisions of each of these four lines was recorded daily, at which time a single organism was isolated in fresh culture medium.   Consequently the rate of division of *Ad2* is the average rate of division of the four lines comprising it.

The culture in which the medium was changed at forty-eight-hour intervals showed that the organisms in a volume of five drops divided 5.3 per cent more rapidly than those in two drops; those in twenty drops divided 9.3 per cent more rapidly than those in two drops, and those in forty drops divided 9.25 per cent more rapidly than those in two drops (fig. 4, B).   The details of each of the four experiments are graphically shown in fig. 3,

which gives the rate of division of each of the lines in the different volumes of medium averaged for each four days of the experiment.

This series of experiments covered seventy-two days and was run simultaneously and coextensively with those already outlined with which it is compared, and all the conditions to which each was subjected were identical, the only factor requiring special mention being the food content of the culture medium. It is stated in the description of the cultures of the Ad series of experiments that the culture medium was made up on alternate days— so that the organisms in this series were continued for two days on infusion that was made up at the same time, but which was supplied fresh (from the stock flask) at the end of the first twenty-four hours. Thus the only difference in the treatment of series Aa and Ad was that the change to a second supply of the culture medium was not made at the end of twenty-four hours. Therefore the only difference in the environment of series Aa and Ad, at the beginning of the second twenty-four hours, was that Ad was put in a fresh portion of the medium in which it had been for the past day, and consequently a medium not contaminated by its own excretion products, whereas Aa was continued for the next twenty-four hours in the same portion of medium in which it had been living for the previous day. It is believed that cross infection rendered the bacterial flora of the infusion in the supply flask and that on the Aa culture slides essentially the same and observation also showed that the supply of bacteria on the culture slides was ample.

The results, then, of this series of cultures in which the organisms were isolated every forty-eight hours, confirms the general result derived from the series isolated every twenty-four hours, i.e., that an increased volume of medium is conducive to more rapid multiplication,[29] *and further it clearly shows that the gain in rate of division*

[29] Attention is called to the fact that Aa-20 gained 0.05 per cent more over Aa-2 than did Aa-40 and therefore is an exception. Cf. fig. 4, B. It is possible that another factor enters, or at least becomes perceptible after twenty-four hours in a volume as large as forty drops. The bacteria in this quantity of culture fluid may
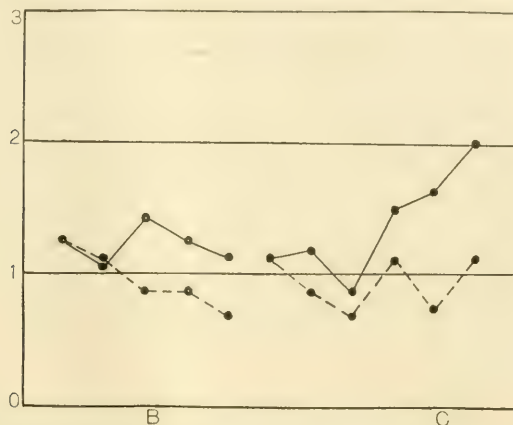
Fig. 5   Record of the experiments on the effects of changing the culture medium at twenty-four-hour intervals and at forty-eight-hour intervals, when Paramaecium aurelia is bred in *two* drops of hay infusion.   The ordinates represent the average daily rate of division of the four lines of organisms, again averaged for four day periods.   Medium changed at twenty-four-hour intervals=———; changed at forty-eight-hour intervals = - - - -.
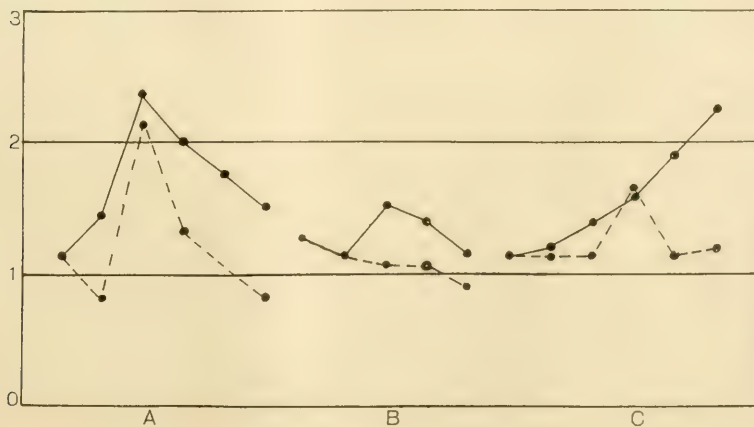

Fig. 6   Record of experiments on the effect of changing the culture medium at twenty-four-hour intervals and at forty-eight-hour intervals, when Paramaecium aurelia is bred in *five* drops of hay infusion.   The ordinates represent the average daily rate of division of the four lines of organisms, again averaged for four day periods.   Medium changed at twenty-four-hour intervals =———; changed at forty-eight-hour intervals = - - - -.

of *Aa-5, Aa–20* and *Aa-40* over *Aa-2 is in every case greater than
the gain of Ad-5, Ad-20* and *Ad-40* over *Ad-2, (cf. fig. 4)*. Again,
from a consideration of the data of comparable cultures changed
daily and that of cultures of equal volumes of media changed on
alternate days, it is found that the gain of the series changed
daily over those changed at fory-eight hour intervals is over
eight per cent in the case of two drops and slightly over six per
cent in the case of five, twenty, and forty drops. Consequently,
as one would expect, changing the medium on alternate days has
most influence in the smallest volume of medium (for details cf.
figs. 5, 6, 7 and 8).

The results of some of the experiments performed, for control
and comparison, on the P. caudatum culture are plotted in fig 9,
and a glance at this will show that they are perfectly consistent
with those derived from the work on P. aurelia. A further check
on the work is also brought out in the first series of experiments
(A) plotted in fig. 9. At the end of the fifth period of this ex-
periment, another culture was isolated, line by line, from *Ca*
which was designated *Cad*, and thereafter in this the medium was
changed daily. The result, as shown in the diagram, was that
the organisms within the following eight days attained the same
division rate as that of the culture *Cd*, thus clearly indicating that
the variation in the division rate between *Cd* and *Ca* was effected
by the duration in time to which they were subjected to the
culture medium.

Further, as a control to test the accuracy of the general method
employed in all the work, a duplicate control culture, designated
Ad-5dup, was carried and the number of divisions in this culture
at the conclusion of the work was exactly the same as *Ad-5*. Of
course it was an 'accident' that there should have been no varia-
tion during the long series of experiments, but this indicates
that such error as exists in the method used is very slight, and

develop so fast that they exhaust their own food and produce excretion products
in sufficient amount to be detrimental to the paramaecia, whereas in the smaller
volumes of medium the animals keep the bacteria reduced so that they do not ex-
haust their food and so continue to multiply, providing food for the paramaecia,
but not sufficient excretion products to have a perceptible effect.

Fig. 7 Record of experiments on the effect of changing the culture medium at twenty-four-hour intervals and at forty-eight-hour intervals, when Paramaecium aurelia is bred in *twenty* drops of hay infusion.   The ordinates represent the average daily rate of division of the four lines of organisms, again averaged for four day periods.   Medium changed at twenty-four-hour intervals= ———; changed at forty-eight-hour intervals = ----.
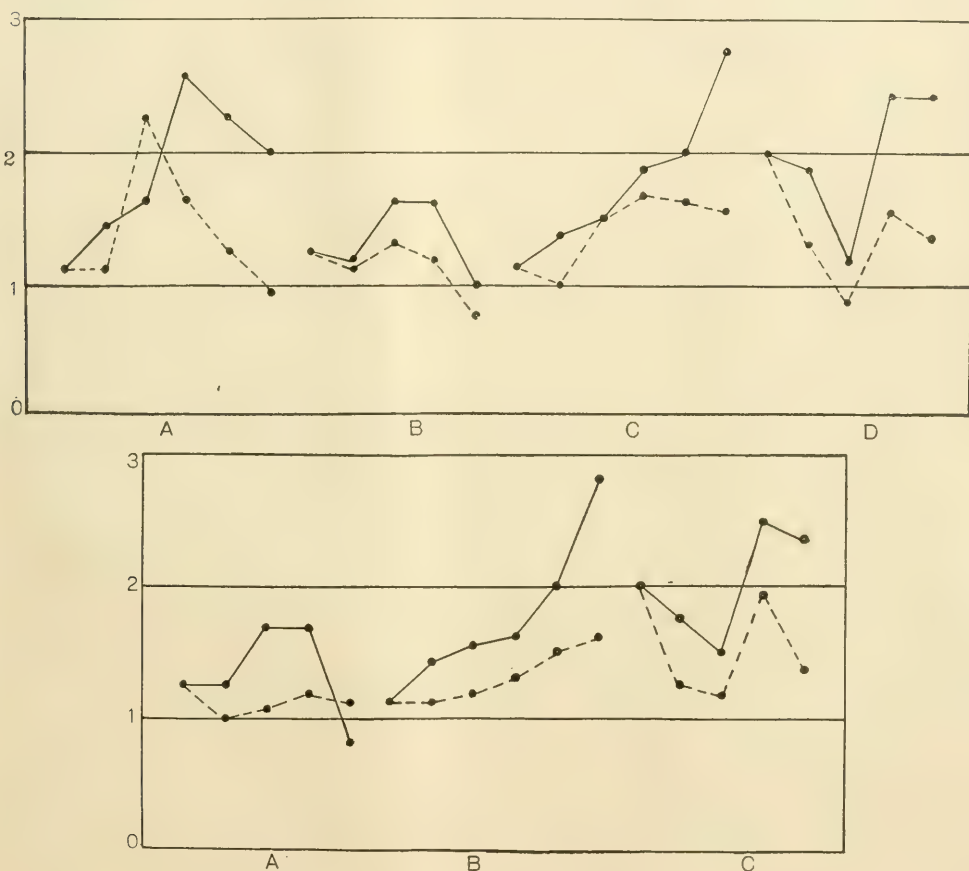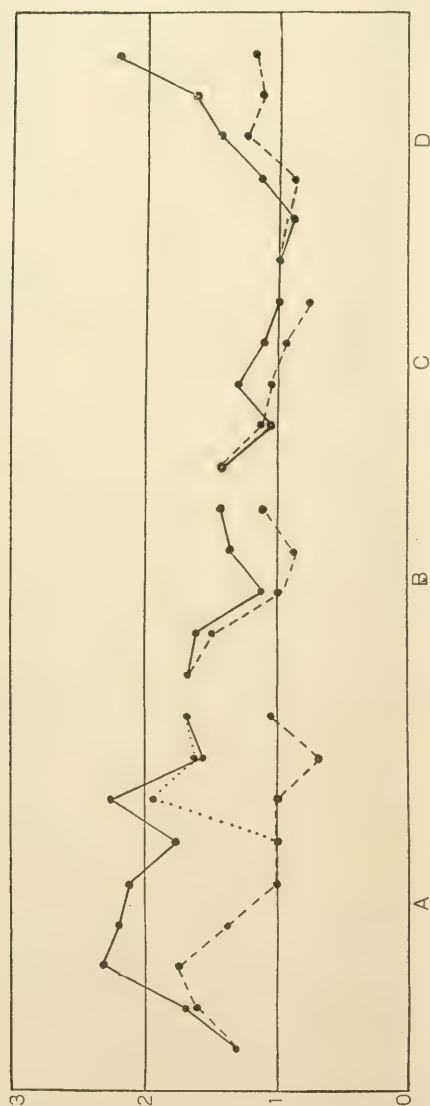

Fig. 8 Record of experiments on the effects of changing the culture medium at twenty-four-hour intervals and at forty-eight- hour intervals, when Paramaecium aurelia is bred in *forty* drops of hay infusion.   The ordinates represent the average daily rate of division of the four lines of organisms, again averaged for four day periods.   Medium changed at twenty-four-hour intervals; = ———; changed at forty-eight-hour intervals = ----.

Fig. 9 Record of experiments on the effect of changing the culture medium at twenty-four-hour intervals and at forty-eight-hour intervals, when Paramaecium caudatum is bred in *five* drops of hay infusion. The ordinates represent the average daily rate division of the four lines of organisms, again averaged for four day periods. Medium changed at twenty-four-hour intervals = ——— and ⋯⋯⋯; changed at forty-eight-hour intervals = - - - - - .

that the differences in the division rate in the experiments are far beyond the limits of error.

It is clear then that all the data derived from the experiments outlined point to the conclusion that *paramaecia excrete substances which are toxic to themselves and that these substances are more effective, as one would expect, when the organisms are confined in limited volumes of culture medium.* The logical method of procedure is to determine the influence of media known to be contaminated with the excretion products of large numbers of paramaecia.

3. *The effect of media in which rich growths of paramaecia have occurred on the rate of reproduction of Paramaecium*

As a culture medium for this experiment an infusion of chopped hay was made and boiled. After a thorough stirring, equal volumes were poured into sterile flasks of a capacity of 250 cc. One of these flasks was then seeded with five cc. of infusion containing twenty-five P. aurelia from the 'stock' left over from the pedigree culture, and the other flask was seeded with five cc. of the same infusion, from which the paramaecia had been removed. The flasks were kept plugged with cotton. There were then two flasks containing precisely the same culture material and bacterial flora, and the one differed from the other only in the presence of paramaecia. After these infusions had stood for ten days there was a heavy growth of paramaecia in one and none in the other, and the media were ready for the experiments. From the four lines of the pedigree culture of P. aurelia two separate cultures were isolated (designated $A+P$ and $A-P$ respectively), one of which was bred on hay infusion from the paramaecia-free flask $(F-P)$, and the other on material from the flask inoculated with paramaecia $(F+P)$. It was necessary, of course, to remove the paramaecia from the culture medium before it was used in the experiments, and this was done daily by filtering it through filter paper before it was used, and then examining it carefully under the microscope, and picking out with a pipet the few animals which had not been retained by the filter paper. As a precau-

tion, the infusion from the flask minus the paramaecia was similarly filtered so that there could be no possibility of error through the filtration process affecting the bacterial content or contaminating the infusion. Further, since in the culture medium $F+P$ the paramaecia had undoubtedly decreased the number of bacteria present through feeding on them, the fluid, after the animals were strained out, was inoculated with bacteria from the medium $F-P$, and as an added precaution $F-P$ was inoculated with bacteria from $F+P$. Thus, while the bacteria were reduced in $F+P$ through being eaten by the animals, this medium undoubtedly contained more food for bacteria, and therefore, when inoculated, should develop bacteria more rapidly than the $F-P$ culture medium, and therefore there would be at least as much food for the organisms on which the experiments were made in $F+P$ as in $F-P$.

It is believed then that, when the media were employed in the experiments, they were practically identical except that one contained the products of metabolism of a heavy growth of paramaecia while the other was absolutely free from such contamination.

The results of this experiment are shown graphically in fig. 10. A shows the results of a preliminary observation which includes the daily isolations for four days. B illustrates the results from another and longer experiment made by the reisolation of both series from the main pedigree cultures A. C gives clearly the same general results which were obtained when the whole experiment was repeated, this time with new flasks of culture medium, etc.

As a further check on the results, fig. 10, B shows that, when after eight days subjection to the $F+P$ medium, another culture was isolated line by line from it and put on the $F-P$ medium, the division rate approached the rate of that continuously on the $F-P$ medium. And again when still another culture was isolated from the culture recently transferred from the $F-P$ medium, and put back on the $F+P$ medium, the rate of division of this new culture approached that of the series which had been continually on the $F+P$ medium.

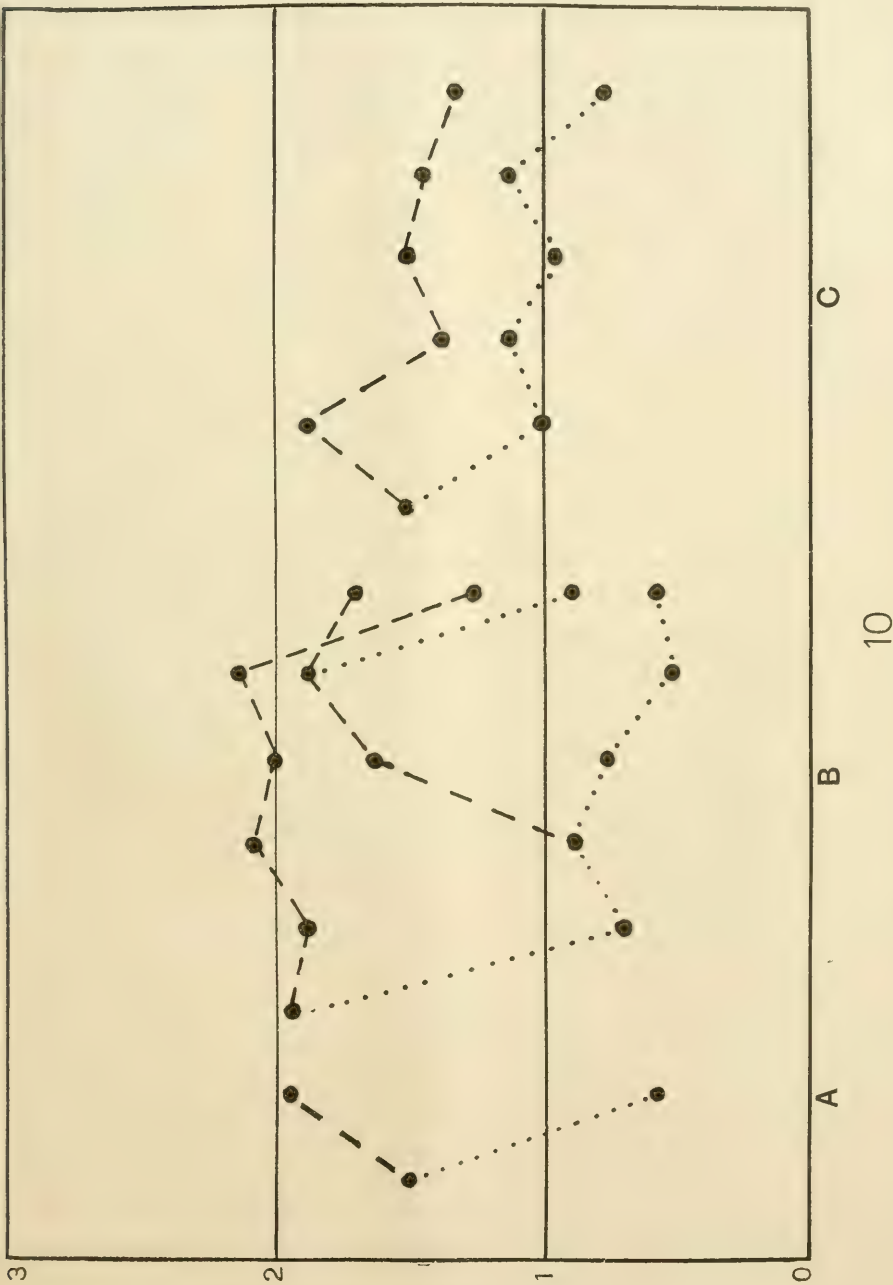A short series of experiments for comparison was made with

Fig. 10 Record of the experiments to determine the effect of a medium, which has supported a rich growth of Paramaecium aurelia, on the rate of reproduction of P. aurelia. The ordinates represent the average daily rate of division of the four lines of organisms, again averaged for four day periods. Rate of division of organisms in culture medium free from the excretion products of Paramaecium (−P) = − − − −, culture medium with excretion products (+P) = ·······

the same two culture media on the pedigree culture of *P.* caud-
atum, and this gave the same general result. In this experiment
the animals on the $F+P$ medium died out (fig. 11).

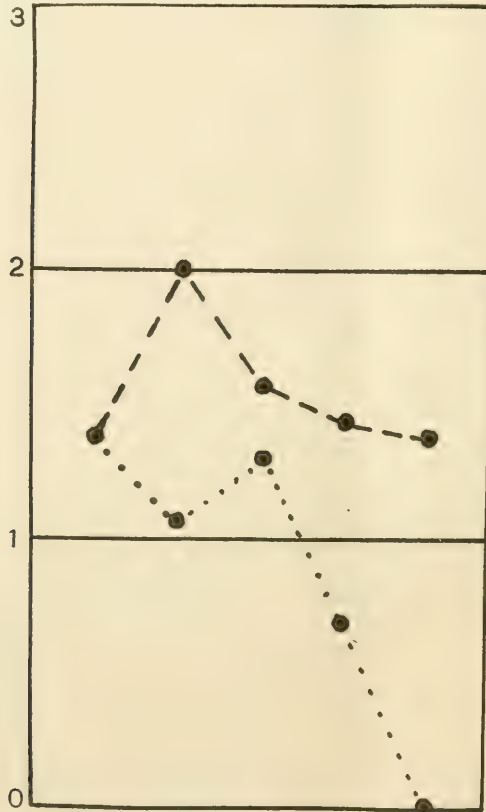It is obvious then from these data that *culture media in which*

Fig. 11. Record of an experiment to determine the effect of a medium, which
has supported a rich growth of Paramaecium aurelia, on the rate of reproduction
of P. caudatum.   The ordinates represent the average daily rate of division of the
four lines of organisms, again averaged for four day periods.   Rate of division
of organisms in culture medium free from the excretion products of P. aurelia
$(-P) = ---$, culture medium with excretion products $(+P) = \cdots$

paramaecia have been living has a decidedly depressing effect on the
rate of reproduction of paramaecia of the same pure pedigree stock
as the contaminating animals, as well as on the rate of reproduc-
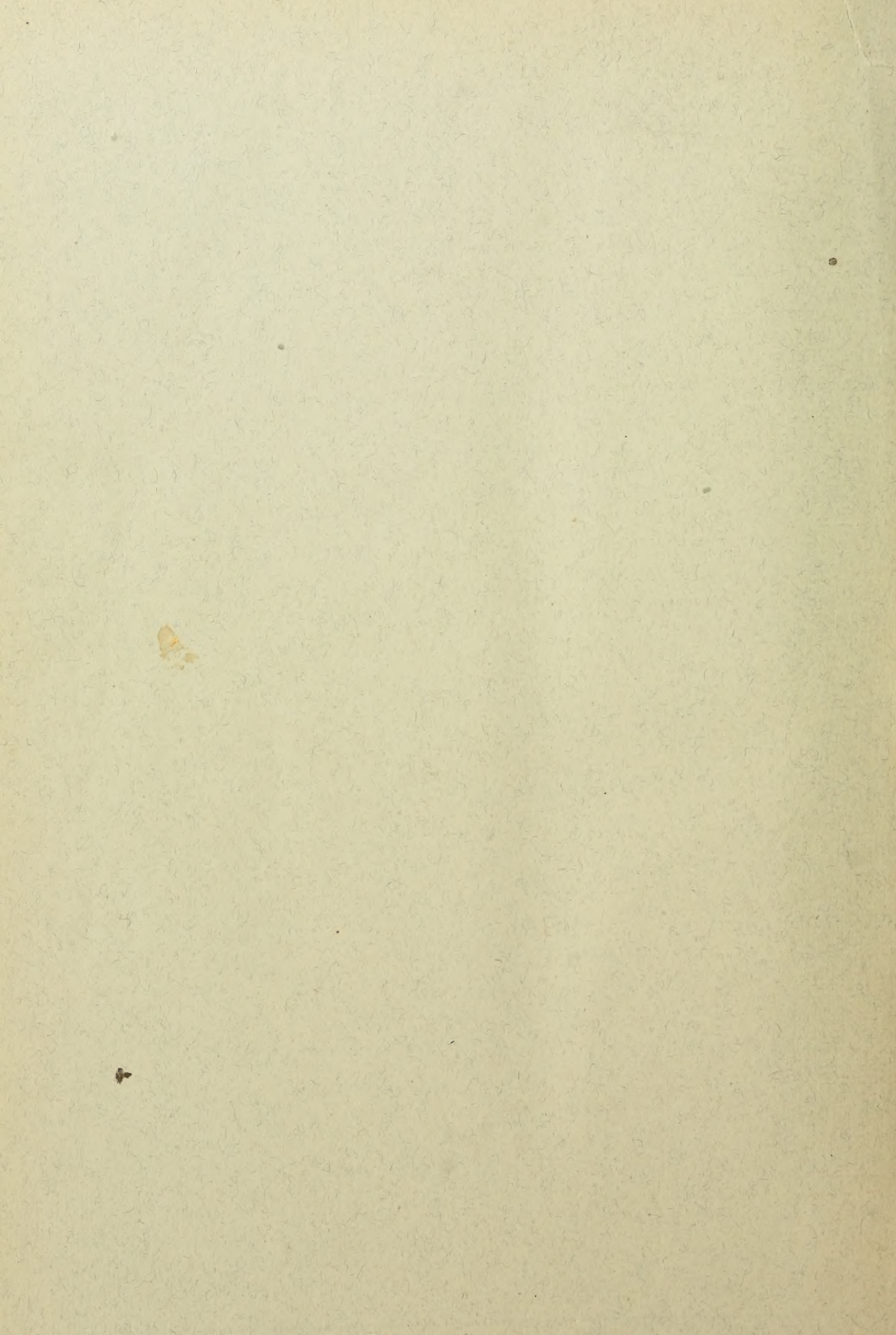tion of another species, P. caudatum.

CONCLUSIONS

In this paper are presented the initial experiments of a series which is planned to elucidate, if possible, some of the complex factors at work in a 'hay infusion,' for example, such as those which determine the interdependence of the organisms, their sequence, time of appearance and disappearance, etc. The data outlined were derived from the study of the following points:

(1) The effect of different volumes of culture medium on the rate of reproduction of Paramaecium; (2), The effect of changing the culture medium daily and on alternate days on the rate of reproduction of Paramaecium; and (3), The effect of culture medium, in which many paramaecia have been living, on the rate of reproduction of Paramaecium. It is believed that the results obtained justify the following conclusions:

1. The rate of reproduction of Paramaecium aurelia and Paramaecium caudatum is influenced by the volume of the culture medium, within the limits tested, and the greater the volume the more rapid is the rate of division.

2. Paramaecia excrete substances which are toxic to themselves when present in their environment, and these substances are more effective when the organisms are confined in limited volumes of culture fluid.

3. The excretion products of paramaecia play an appreciable part in determining the period of maximum numbers, rate of decline, etc., of this animal in 'hay infusions.'

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